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RhoA-ROCK Downregulates Akt phosphorylation through PTEN in Cells Poorly Attached to Substrates

부착결핍 세포에서 PTEN 매개에 의한 RhoA-ROCK의 Akt 인산화 억제에 관한 연구

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양 승 원

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ABSTRACT

RhoA-ROCK Downregulates Akt phosphorylation through PTEN in Cells Poorly Attached to Substrates

Seungwon Yang

Department of Cell and Developmental Biology

School of Dentistry

The Graduate School

Seoul National University

(Directed by Prof. Hyun-Man Kim, D.D.S., Ph.D.)

Survival and proliferation of anchorage-dependent cells such as osteoblasts and fibroblasts require an attachment of cells to substrates. Thus, cells that poorly attached to substrates exhibit retarded cell cycle progression or apoptotic death. This study investigated the molecular mechanism underlying the reduced proliferation rate of the cells that poorly attached to the substrates. There was an inverse relationship between the activity of the small GTPase RhoA (RhoA) and the cell proliferation rate. The RhoA activity correlated inversely with the

strength of cell adhesion to the substrates. The high RhoA activity in the cells poorly attached to substrates caused an increase in the activity of Rho-associated kinase (ROCK), a well-known effector of RhoA that upregulated the activity of phosphatase and tensin homolog (PTEN). The resulting activated PTEN downregulated Akt activity, which is essential for cell proliferation. Thus, the cells that poorly attached to substrates showed low levels of cell proliferation because the RhoA-ROCK-PTEN pathway was hyperactive. In addition, RhoA activity seemed to be related to focal adhesion kinase (FAK) activity. Weak FAK activity in the poorly attached cells failed to downregulate the high RhoA activity that restrained cell proliferation. Interestingly, reducing the activity of any component of the RhoA-ROCK-PTEN pathway rescued the proliferation rate without physicochemical surface modifications. Based on these results, this study suggests that the RhoA-ROCK-PTEN pathway acts as a molecular switch to control cell proliferation and determine anchorage dependence.

KEYWORDS : cell adhesion, anchorage-dependence, cell proliferation, cell signaling

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I. INTRODUCTION

1. Biological and clinical significance of cell adhesion signaling

Cell adhesion signaling is induced and maintained through attaching to extracellular matrix or adjacent cells. Adhesion-mediated signaling is required for cell survival, migration, proliferation and differentiation [1-3]. Especially, cells cannot grow and divide without adequate adhesion signaling, which is called anchorage dependence. Indeed, adhesion signaling is necessary for a cell to progress into cell cycle and growth factor-dependent signaling alone cannot induce cell cycle progression on a substrate where cell-adhesion signaling is weak [4]. Osteoblasts do not proliferate well on the thin film of calcium phosphate apatite because growth factor signaling and adhesion signaling do not adequately cross-talk to activate the mitotic signaling pathway [5]. Even in the presence of strong growth factor stimulation, osteoblasts proliferate slowly on the calcium phosphate apatite surface where osteoblasts develop poor stress

fibers and focal adhesions owing to insufficient activation of cell adhesion signaling pathways.

Cell signaling pathways associated with cell adhesion have been among the most studied topics in the fields of medicine and related area. In the field of medicine, for instance, cell adhesion molecules and their related down-stream pathways are important drug targets in cancer [6, 7]. Integrins regulates behavior of tumor cell such as adhesion, migration, invasion and survival. Resistance to anoikis, inadequate cell adhesion-induced apoptosis, is one of the most important factors in metastatic cancer processes [8-12]. In addition, improving cell attachment to scaffold through facilitating cell adhesion to extracellular matrix proteins has been one of hot topics in the area of tissue engineering [13]. Anchorage-dependent cells such as osteoblasts proliferate slowly or die by anoikis if adequate cell attachment to the extracellular matrix is not provided to generate an appropriate adhesion signaling cascade [4, 14, 15]. Hydrophobic surfaces are typical substrates on which cells cannot

acquire adequate adhesion signaling because the cells and substrates are weakly attached [4, 14]. Thus, the hydrophobicity of most scaffolds used for tissue engineering, such as poly(lactic-co-glycolic acid) and poly(ε-caprolactone), is one of the major obstacles faced when cells grow on such polymeric biomaterials [16].

2. Intracellular transduction of cell adhesion signaling

Cell adhesion signal transduction is triggered by clustering adhesion molecules such as integrins and cadherins when adhesion molecules on cell membrane recognize specific adhesion ligands on extracellular matrix or adjacent cells [17-19]. Integrins, major adhesion molecules, are known as transmembrane receptors and dynamically link to actin cytoskeleton [20, 21]. Integrins provide the communication between the cells and their surrounding environment such as extracellular matrix or other cells through modulating outside-in or inside-out signal pathway [22, 23]. These processes play important roles in regulating cellular

shape, migration and cell cycle as well as cell survival and differentiation.

It has been reported that integrins clustering during the early cell adhesion activates small GTPase RhoA (RhoA), thereby Rho-associated kinase (ROCK) is upregulated. The activation of RhoA-ROCK signal pathway is essentially required for the formation of focal adhesion and stress fiber, which are the typical markers of appropriate cell attachment to substrates [24-27]. ROCK is known to induce the formation of stress fibers and focal adhesions by deactivating myosin light chain phosphatase (MLCP) and phosphorylating myosin light chain [28-30] and to stabilize actin filaments and increases their number by phosphorylation-dependent activation of LIM kinase (LIMK) [31]. Then, it is generally believed that cell proliferation is directly related to the extent of cell spreading and dependent on the developments of stress fibers and focal adhesions which is activated by ROCK.

3. Purpose of the study

Different from the general belief that cell proliferation is closely associated with development of stress fibers and focal adhesions of which development is upregulated by ROCK [24-27], it has been reported that proliferation and migration of osteoblasts were markedly improved after Rho-associated kinase (ROCK) inhibition on hydrophobic surfaces although its molecular mechanism is not known [14]. The ROCK signaling molecule was shown to have anti-apoptotic effects in human embryonic stem cells [32]. In these stem cells that grow in suspension and where adhesion signaling is null, ROCK inhibition enhances their survival and efficiency of the embryonic stem cell clonal expansion [32]. From these previous reports, it is hypothesized that activation of ROCK may inhibit the proliferation of the anchorage-dependent cells with poor adhesion signaling and conversely, inhibition of ROCK may rescue the low proliferation of the cells in which adhesion signaling is poor. Thus, this study was designed to confirm such hypothesis by investigating the mechanism by which the poor adhesion of cells to substrates activates

ROCK and related signaling pathways to upregulate cell proliferation. It would be important to understand anchorage dependence of metastatic cells in cancer biology and of stem cells in tissue engineering.

II. MATERIALS AND METHODS

1. Model substrates

For this study, various surface conditions were applied. Hydrophobic substrate and calcium phosphate apatite substrate were used for culturing cells on the surface condition of weak cell adhesion. Poly-HEMA substrates were used for suspension culture. Hydrophilic tissue culture plates were used for positive control.

Polystyrene cell culture dishes (Corning, Corning, NY) with water contact angles of 56° were used for culturing cells on hydrophilic (HPL) substrates. Polystyrene bacteriological dishes (SPL Life Sciences, Seoul, Korea) with water contact angles of 85° were used for culturing cells on hydrophobic (HPB) substrates.

Polyhydroxyethyl methacrylate (poly-HEMA) plates were used for culturing cells in suspension. A solution of poly-HEMA (Sigma-Aldrich,

St. Louis, MO) mixed in ethanol was poured onto polystyrene bacteriological dishes. After the poly-HEMA had dried, the same procedure was repeated once followed by extensive washing with phosphate buffered saline (PBS) [1, 33].

Transparent thin films of poorly crystalline calcium phosphate apatite (PCA) were prepared using previously described methods [5, 34, 35]. Briefly, phosphate-buffered solution (PBS, pH 7.2) containing 2.5 mM Ca^{2+} prepared at 1° C was poured into untreated polystyrene culture dishes (Corning, Corning, NY) at 1° C. Then the dishes were placed in a 42° C incubator for 35-45 min to coat the surfaces with a PCA thin film and used for cell culture after sterilization with 70% ethanol. The PCA thin films were observed using field emission-scanning electron microscopy (FE-SEM) (S-4700, Hitachi, Tokyo, Japan) after sputter-coating with gold-palladium. The physico-chemical properties of the PCA thin film have been described in detail previously in Ref. [5, 34, 35].

2. Cell culture and reagents

Mouse pre-osteoblast MC3T3-E1 cells (clone 4; ATCC, Manassas, VA) were cultured in α-MEM (WelGENE Inc., Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Carlsbad, NY), 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO BRL, Carlsbad, NY) and were grown at 37 ° C in 95% air and 5% CO2. MC3T3-E1 cells were differentiated into functional osteoblasts by culturing them in osteogenic α -MEM containing 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO), 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO). The following specific pharmacological reagents were used to regulate cell signaling: LY-294002 (Sigma-Aldrich, St. Louis, MO) for phosphatidylinositol 3-kinase (PI3K) inhibition, cell-permeable C3 transferase (C3) (Cytoskeleton, Denver, CO) for RhoA inactivation, Pasteurella multocida toxin (PMT) (Sigma-Aldrich, St.Louis, MO) and lysophosphatidic acid (LPA) (Sigma-Aldrich, St. Louis, MO) for RhoA

activation, potassium bisperoxo(1,10-phenanthroline)oxovanadate (bpV(Phen)) (Calbiochem, La Jolla, CA) for phosphatase and tensin homolog (PTEN) inhibition, Y-27632 (Tocris Cookson, Avonmouth, UK) for ROCK inhibition, and FAK inhibitor 14 (Tocris Cookson, Avonmouth, UK) for focal adhesion kinase (FAK) inhibition. The HPB substrates were precoated with small amounts of fibronectin (Sigma-Aldrich, St. Louis, MO), an adhesion ligand, to facilitate the transfection of siRNA or various constructs.

3. Cell proliferation assay

The numbers of MC3T3-E1 cells that grew for the determined time periods were assessed with the CCK-8 proliferation assay (Dojindo, Kumamoto, Japan). The CCK-8 analysis was performed following the manufacturer's protocol.

4. Immunoblotting

The cells were lysed with a lysis buffer (Cell Signaling Technology, Beverly, MA) composed of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF and a complete protease inhibitor cocktail tablet (Santa Cruz Biotechnology, Santa Cruz, CA). After high-speed centrifugation of the cell lysates, the protein concentrations of the supernatants were determined by the Bradford assay (Bio-Rad, Hercules, CA). The proteins were run on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in TBS-T buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20). They were incubated with primary antibodies overnight at 4 ° C and then with secondary antibodies in 5% skim milk in TBS-T for 1 h at room temperature. The blots were developed using the horseradish peroxidase (HRP) chemiluminescent substrate reagent kit (Invitrogen, Carlsbad, CA).

The following antibodies were used: phospho-AKT (Ser 473) (Cell Signaling Technology, Beverly, MA), AKT (Cell Signaling Technology, Beverly, MA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA), RhoA (Cell Signaling Technology, Beverly, MA), PTEN (Cell Signaling Technology, Beverly, MA), phospho-PTEN (Ser380, Thr382/383) (Cell Signaling Technology, Beverly, MA), phospho-Paxillin (Tyr118) (Cell Signaling Technology, Beverly, MA), FAK (Cell Signaling Technology, Beverly, MA), phospho-FAK (Tyr397) (Cell Signaling Technology, Beverly, MA), ROCK-1 (Cell Signaling Technology, Beverly, MA), RhoA (Cytoskeleton, Denver, CO), phospho-non-muscle myosin light-II (ser 19) (Millipore Co., Bedford, MA), PTEN (Santa Cruz Biotechnology, Santa Cruz, CA), ROCK-1 (BD Biosciences, San Diego, CA), bete-actin (Cell Signaling Technology, Beverly, MA), $p27^{Kip1}$ (Cell Signaling Technology, Beverly, MA), $p21^{Waf1/cip1}$ (Cell Signaling Technology, Beverly, MA), Cyclin D1 (Calbiochem, La Jolla, CA) and HRP-conjugated secondary antibodies

(Cell Signaling Technology, Beverly, MA).

5. Confocal laser microscopic observation

The cells were fixed with 4% paraformaldehyde in PBS for 30 min and then permeabilized in 0.2% Triton X-100 in PBS for 20 min. After the cells were blocked with 2% bovine serum albumin in PBS, the actin filaments were stained with rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA). The stained cells were observed with confocal laser microscopy (Olympus FV-300, Tokyo, Japan).

6. RhoA activity assay

The level of GTP-loaded RhoA was determined using the G-LISA RhoA activation assay kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions. Because the expression level of RhoA varied depending on the type of substrate, equal amounts of proteins from each experimental group were used in the G-LISA RhoA activation assay to

obtain values for the total amount of RhoA activity per cell.

7. Immunoprecipitation

The cells were lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF and a complete protease inhibitor cocktail tablet. The cell lysates were subject to high speed centrifugation. The supernatants were incubated with an antibody and 20 ml of protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4 ° C. The immunocomplexes were pelleted and washed three times with cold lysis buffer. The proteins were released from the beads by boiling in SDS sample buffer. The samples were analyzed by immunoblotting methods.

8. Transient transfection and siRNA transfection

MC3T3-E1 cells were seeded on the HPB substrates coated with fibronectin (50 ng/ml) overnight prior to the transient transfection of each plasmid using the PolyJet^{TM} in vitro DNA transfection reagent (Signagen, Ijamsville, MD) according to manufacturer's instructions. The expression of each plasmid was confirmed by cell shape changes, immunoblot analyses, or fluorescence analyses. The transfection efficiency was approximately 40-50% (data not shown). The following plasmids (Addgene, Cambridge, MA) were used: pCDNA3-EGFP (Addgene ID 13031), pCDNA3-EGFP-RhoA-Q63L (constitutively active RhoA, Addgene ID 12968), pCDNA3-EGFPRhoA- T19 N (dominant negative, Addgene ID 12967), and pCDNA3-GFP-PTEN (Addgene ID 10759). For RNA interference (RNAi) experiments, MC3T3-E1 cells were seeded onto the HPL substrates or the HPB substrates, which were coated with fibronectin (50 ng/ml) to promote minimal adhesion while inhibiting cell proliferation, and the cells were then allowed to settle and grow overnight. The cells were transfected

with siRNAs for RhoA, ROCK1, PTEN, and FAK (Santa Cruz Biotechnology, Santa Cruz, CA) using siRNA transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA) according to manufacturer's instructions.

9. Caspase-3 activation assay

The caspase-3 activity was determined using a ApoAlert Caspase-3 Colorimetric Assay Kit (Clontech, Palo Alto, CA).

10. Cell viability assay

Calcein AM solution (2 μM)(Sigma, St. Louis, MO) and Ethidium homodimer(1 μM) (Sigma, St. Louis, MO) were used for analyzing viability of cells. Each solution was co-treated in cell culture medium and was incubated for 30-60 min.

11. Statistical analysis

Each experimental value represents the mean ± the standard deviation of at least three samples. The statistical analyses were performed with the t-test or one-way analysis of variance with posthoc multiple comparisons. A P-value of <0.05 was considered statistically significant.

III. RESULTS

1. Cells on HPB substrates

1.1. Anchorage dependence of cell growth

The cells cultured on the hydrophobic bacteriological culture plates showed similar characteristics to cells grown on substrates that generate weak cell adhesion signaling. The cells cultured on HPB substrates spread less and proliferated slowly. Compared with the cells cultured on the hydrophilic cell culture plates, the cells grown on the bacteriological culture plates exhibited low phosphorylation levels of FAK, which is a key signaling molecule associated with cell adherence to substrates [36, 37], and low phosphorylation levels of AKT, which is involved in cell cycle progression [38, 39] [Fig.1 A–D]. This dependence of cell growth on anchorage was further confirmed when the plates were precoated with various amounts of fibronectin to vary the strength of cell adhesion. The rate of proliferation and the phosphorylation levels of FAK and AKT directly correlated with the amount of fibronectin that was used to coat

the substrates before the cells were plated [Fig. 1 E–G]. These results indicate that low activity of the cells cultured on the HPB substrates is caused by the low adhesion strength to the HPB substrates.

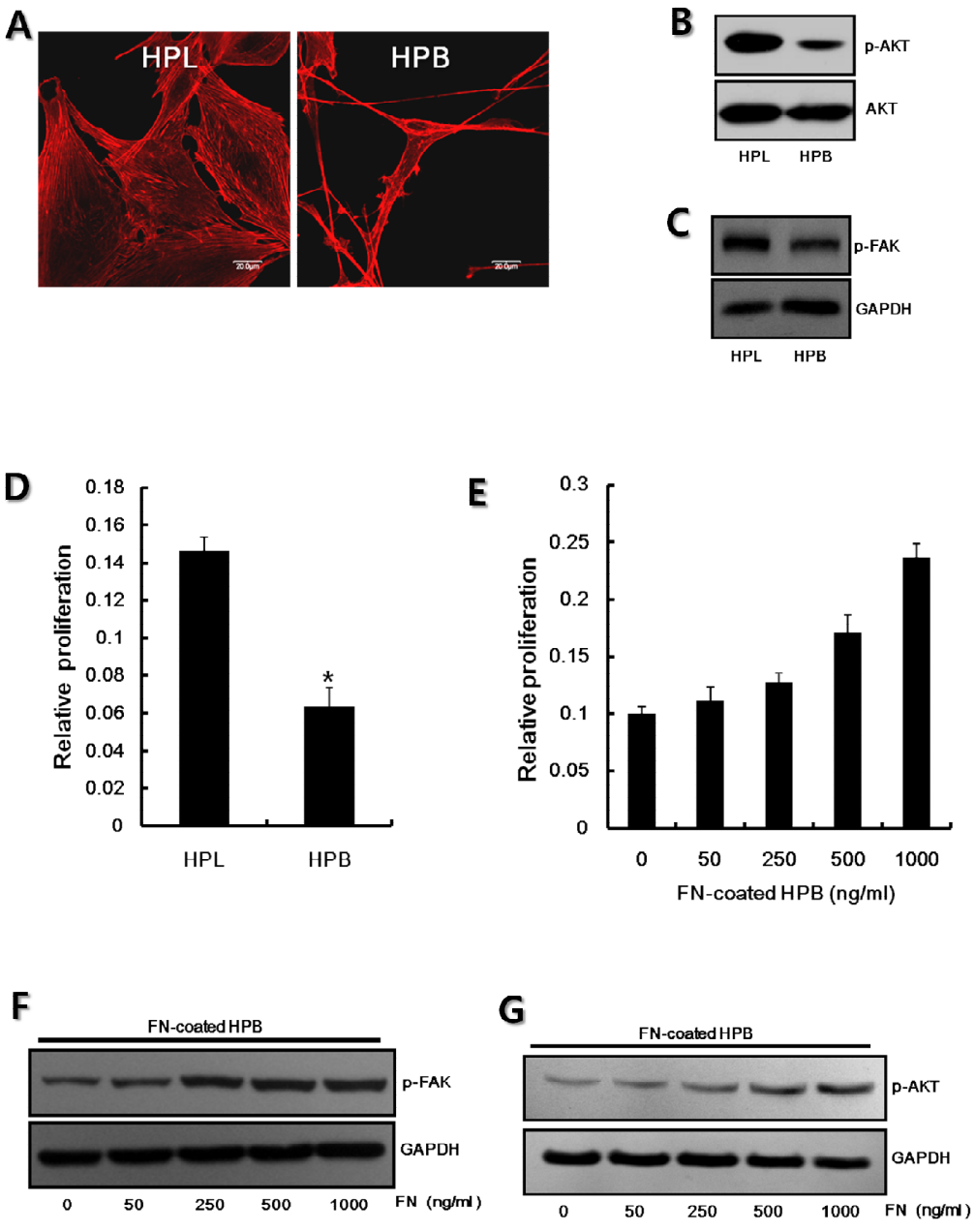


Fig. 1 Activity of MC3T3-E1 cells is dependent on adhesion strength.

Cell activity was examined in the cells grown on hydrophobic bacteriological culture plates (HPB) with weak adhesion strengths or hydrophilic cell culture plates (HPL) with high adhesion strengths (A – D). Cell activity was also examined in the cells grown on HPB substrates precoated with fibronectin in various amounts to provide the cells with different adhesion strengths (E – F). (A) Cells were cultured on the HPL substrates or HPB substrates for 24 h. The fixed cells were treated with rhodamine-phalloidin to examine the development of actin filaments under confocal laser microscopy (red). (B – C) Cells were grown on the HPL or HPB substrates for 24 h. The levels of phosphorylated FAK or Akt were assessed by immunoblotting using specific antibodies. (D) Cells were cultured on the HPL or HPB substrates for two days. Relative cell proliferation rates were then determined by the cell number generated by the cell counting kit (CCK-8). The data are expressed as the mean ± SD. n = 3 culture dishes. *, $P < 0.05$ compared with cells grown on HPL substrates. (E) Cells were cultured for two days on the HPB dishes that

were precoated with the indicated amounts of fibronectin (FN). Substrates were blocked with albumin (5% v/v) after being coated with fibronectin. Relative cell proliferation rates were then determined by the cell number generated by the cell counting kit (CCK-8). The data are expressed as the mean ± SD. n = 3 culture dishes. $P < 0.05$ when all of the cells grown on the FN-coated HPB, except for those grown on 50 ng/ml FN-coated HPB, were compared with the control cells grown on plates without fibronectin coating. (F - G) Cells were grown for 24 h on the HPB dishes that were precoated with the indicated amounts of fibronectin. The phosphorylation levels of FAK (F) and Akt (G) in cells at various adhesion states were assessed by immunoblotting analysis using specific antibodies. The level of GAPDH was assessed to confirm an equal loading of cell lysate.

1.2 RhoA and ROCK in the anchorage dependence of cell growth

Consistent with the conjecture that the RhoA−ROCK−PTEN pathway may reduce the proliferation rate of poorly attached cells, the activity of RhoA was higher in the cells slowly proliferating on the HPB substrates than those normally proliferating on the HPL substrates [Fig. 2A]. The expression level of RhoA was also upregulated in the cells cultured on the HPB substrates [Fig. 2B]. An inverse relationship between RhoA activity and the cell adhesion strength further confirmed that cell adhesion to the substrates suppresses RhoA activity [Fig. 2C]. The expression level of RhoA also increased with decreasing amounts of fibronectin that had been precoated onto the substrates [Fig. 2D]. Next, the activity of ROCK was examined. ROCK is a well−known downstream effector of RhoA [28, 40], by assessing the phosphorylation level of myosin light chain (MLC) [28]. Consistent with the assumption, MLC phosphorylation levels were higher in the cells cultured on the HPB substrates compared with the cells cultured on the HPL substrates, indicating that ROCK activity is higher in the cells that were weakly

attached to the substrates [Fig. 2E]. The expression level of ROCK was also upregulated in the cells cultured on the HPB substrates compared with the cells on the HPL substrates [Fig. 2F]. These results indicate that RhoA-ROCK is activated in the cells that are poorly attached to the substrates.

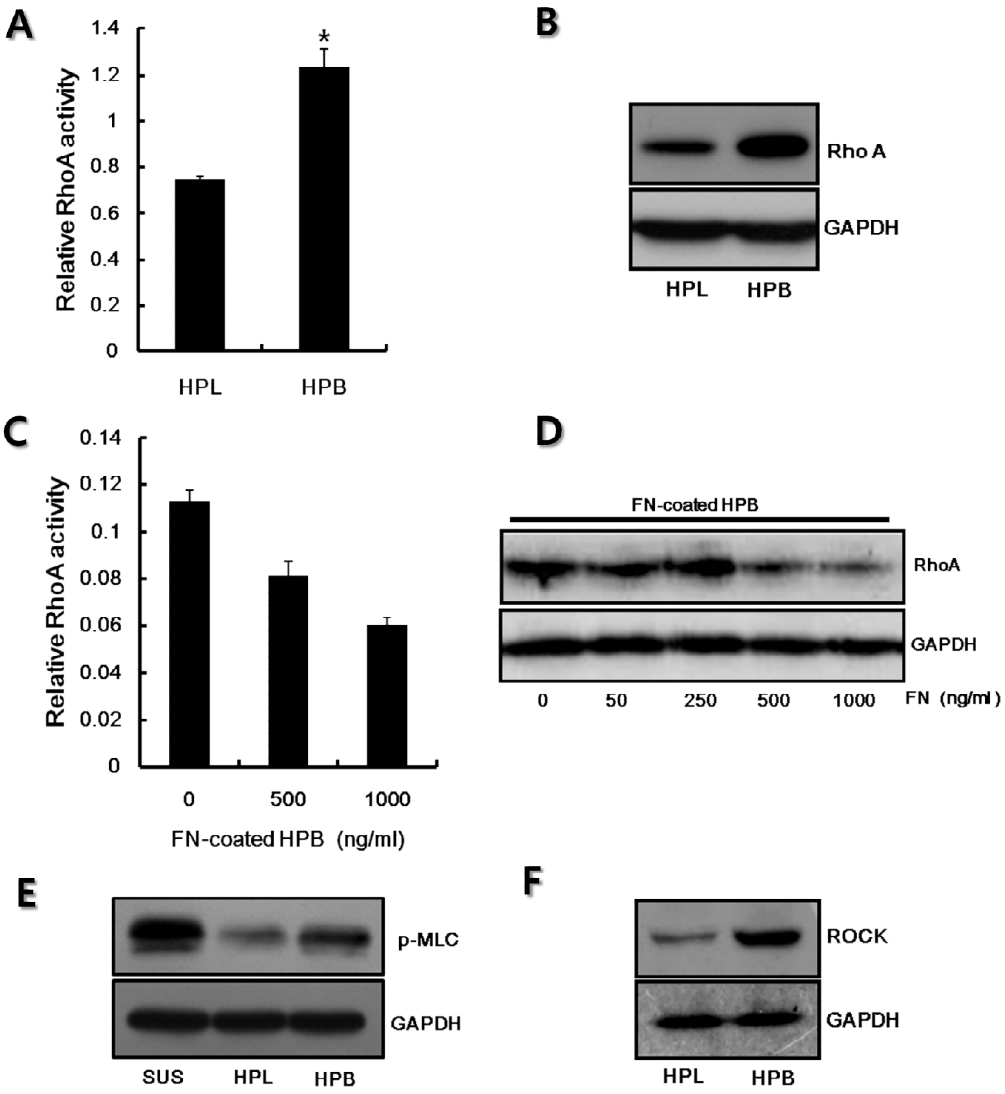


Fig. 2 Expression and activity of RhoA and ROCK are upregulated when cell adhesion to the substrate is weak.

(A) The total cellular RhoA activity of MC3T3-E1 osteoblasts was determined in the cells cultured on HPL or HPB substrates for two days. Equal amounts of cell lysate were used for the G-LISA RhoA activation assay. The data are expressed as the mean ± SD. n = 3 culture dishes. *, $P < 0.05$ when compared with cells grown on HPL substrates. (B) The cells were grown on HPL or HPB dishes for two days, and then RhoA expression levels were determined by immunoblotting using the anti-RhoA antibody. (C) The cells were grown for two days on HPB substrates precoated with the indicated amounts of fibronectin (FN). Total cellular RhoA activity of cells was determined with the G-LISA RhoA activation assay with equal amounts of cell lysate. The data are expressed as the mean ± SD. n = 3 dishes. The p-value was less than 0.05 for comparisons between any two groups. (D) The cells were grown on the HPB substrates precoated with the indicated amounts of fibronectin. RhoA expression levels were determined in the cells cultured for two days by immunoblotting using the anti-RhoA antibody. (E) ROCK

activity was determined by assessing levels of phospho-myosin light chain (p-MLC). The cells were cultured on poly-HEMA substrates for suspension culture (SUS), HPL, or HPB substrates for 3 h. The expression levels of p-MLC were determined by immunoblotting using a specific antibody raised against phospho-non-muscle myosin-light chain II. (F) The cells were grown on the HPL or HPB substrates for two days, and then ROCK expression was determined by immunoblotting using the anti-ROCK antibody. GAPDH levels were monitored for equal loading of the cell lysate (B and D - F).

Next, the activities of RhoA and ROCK were inhibited to determine if their activities are sufficient to regulate adhesion-dependent cell proliferation. The inhibition of ROCK by a pharmacological inhibitor, Y-27632, reversed the low proliferation rate of the cells cultured on the HPB substrates [Fig. 3A. Interestingly, the inhibition of ROCK did not further increase the proliferation rate of the cells cultured on the HPL substrates, where cells had proliferated rapidly. On the HPB substrates, the inhibition of RhoA by the specific inhibitor C3 rescued the normal proliferation rate of the cells, whereas the activation of RhoA by the specific activator PMT further decreased the proliferation rate of the cells [Fig. 3B]. These results indicate that a low activity of RhoA-ROCK is a prerequisite for cellular proliferation and that activation of RhoA-ROCK limits the proliferation rate. The cells on the HPB substrates spread without the development of stress fibers after treatment with Y-27632 [Fig. 3C and D].

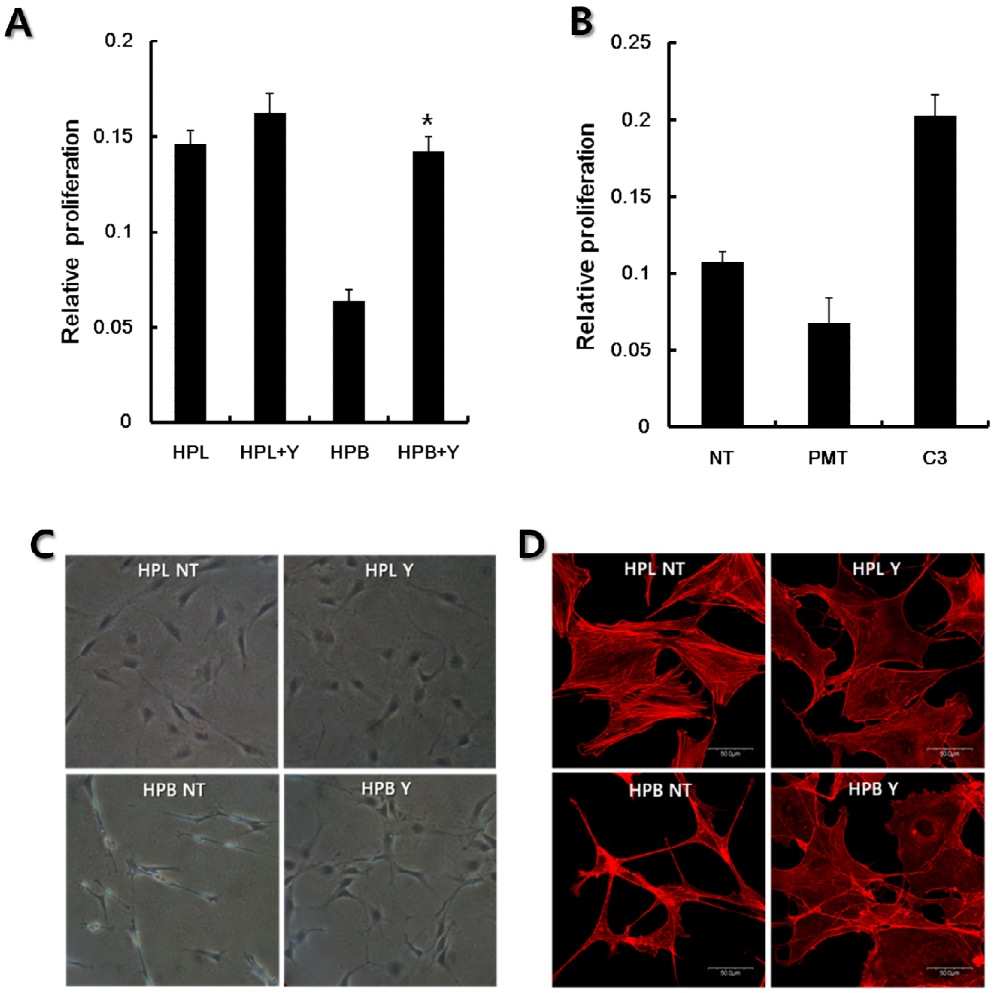


Fig. 3 Inhibition of RhoA or ROCK increases the proliferation rate of MC3T3-E1 cells on the HPB substrates.

(A) The cells were grown on HPL or HPB substrates in the absence or

presence of Y- 27632 (10 μM) to inhibit ROCK activity. The relative cell proliferation rates were determined by the cell number generated with the cell counting kit (CCK-8). The data are expressed as the mean ±SD. n = 3 dishes. *, $P < 0.05$ when compared with the untreated HPB group. (B) The cells were grown for two days on the HPB substrates in the presence of cell-permeable exoenzyme C3 (100 ng/ml) to inhibit RhoA or Pasteurella multocida toxin (PMT) (50 ng/ml) to activate RhoA. The relative cell proliferation rates were determined by assessing the cell number with the cell counting kit (CCK-8). The data are expressed as the mean ± SD. n = 3 dishes. The p-value was less than 0.05 for comparisons between any two groups. (C and D) The cells were cultured on the HPL or HPB substrates in the absence or presence of Y-27632 (10 μM) to inhibit ROCK for 24 h. The cells were observed by phase contrast microscopy (C). Fixed cells were treated with rhodamine-phalloidin to examine development of actin filaments (red) under confocal laser microscopy (D).

Next, the AKT phosphorylation levels were examined in response to the variation in RhoA or ROCK activity because AKT phosphorylation is necessary for cell cycle progression [38, 39]. Concomitant with the supposition, the AKT phosphorylation levels, which were low in the cells grown on the HPB substrates, were increased when RhoA activity was inhibited by transfection of cells with siRNA targeting RhoA or C3 treatment. AKT activity was upregulated upon the overexpression of a dominant negative form of RhoA [Fig. 4A–C]. In contrast, the transfection of cells with constitutively active RhoA and the activation of RhoA by PMT treatment downregulated AKT phosphorylation [Fig. 4A and C]. The inhibition of ROCK activity with Y–27632 treatment or the transfection of siRNA specific for ROCK also increased AKT phosphorylation in the cells cultured on the HPB substrates [Fig. 4C and D]. These results indicate that activated RhoA–ROCK downregulates AKT phosphorylation when adhesion of the cells to the substrate is poor.

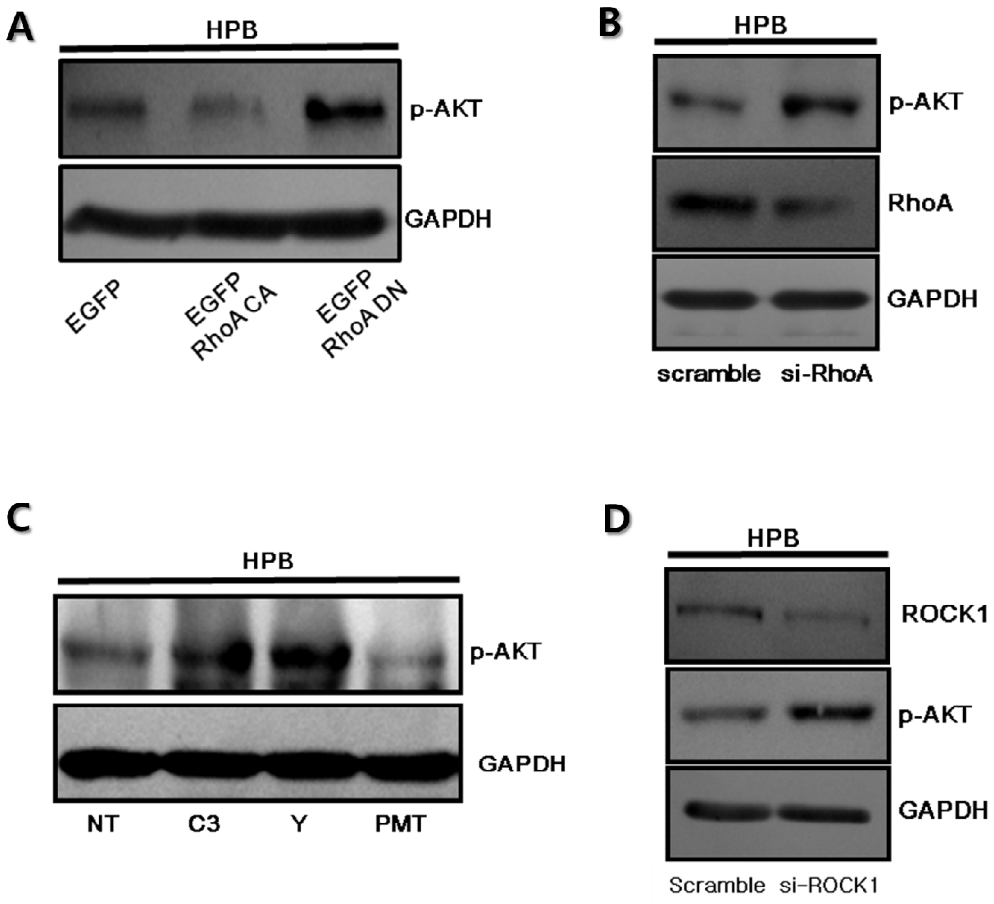


Fig. 4 Inhibition of RhoA or ROCK reverses the low phosphorylation levels of Akt in MC3T3-E1 cells grown on the HPB substrates.

(A) The cells were transiently transfected with pCDNA3-EGFP (transfection control), pCDNA3-EGFP-RhoA T19N (dominant negative

RhoA), or pCDNA3-EGFP-RhoA Q63L (constitutively active RhoA) and further cultured for 24 h. Then, the cell lysate of each group was immunoblotted to assess phosphorylation levels of Akt using the anti-p-Akt antibody. (B) Akt phosphorylation of cells grown on the HPB substrates for 48 h after transfection with control siRNA (scramble) or with siRNA targeting RhoA (si-RhoA) was assessed. (C) The cells were cultured on the HPB substrates for two days. The level of Akt phosphorylation was assessed after the sample groups were treated with Y-27632 (10 μM), C3 (200 ng/ml), or PMT (50 ng/ml) for 3 h. NT indicates the non-treated group. (D) Akt phosphorylation of cells grown on the HPB substrates for 48 h after transfection with control siRNA (scramble) or siRNA targeting ROCK (si-ROCK) was assessed. (A - D) Growth factor signaling was activated by treating cells with fibroblast growth factor 2 (FGF2) (50 ng/ml) to activate Akt for 5 min before harvesting the cell lysate. GAPDH levels were monitored for equal loading of cell lysate.

1.3. PTEN in the anchorage dependence of cell growth

Recently, ROCK has been reported to regulate PTEN to control cell migration [41, 42]. However, the mechanisms for the control of PTEN by cell adhesion remain unclear. Therefore, in this study, it was investigated whether PTEN is a downstream effector of RhoA-ROCK that regulates AKT phosphorylation in cells that weakly adhere to the HPB substrates. PTEN activity, which can be assessed at the expression and phosphorylation levels [41-45]was upregulated in the cells grown on the HPB substrates when compared with those on the HPL substrates [Fig. 5A]. It was further confirmed that this inverse dependence of PTEN activity on the strength of cell adhesion by observing a decreasing level of PTEN activity according to the increase in the amount of fibronectin, which was precoated at varying doses onto the substrate surfaces to increase the cell adhesion strength [Fig. 5B]. The inhibition of RhoA by siRNA or the transfection of cells with a dominant negative form of RhoA also downregulated the activity of PTEN, which was upregulated in the

cells cultured on the HPB substrates. The activation of RhoA by the transfection of cells with constitutively active RhoA upregulated the activity of PTEN [Fig. 5C and D]. In addition, the activity of PTEN was downregulated after ROCK activity was inhibited by treatment with Y-27632 [Fig. 5E]. The activation of PTEN by ROCK seems to occur by direct association of two molecules. The immunoprecipitation results showed that PTEN and ROCK associated more in the cells cultured on the substrates with lower adhesion strength, such as HPB substrates, than in cells cultured on HPL substrates [Fig. 5F] and substrates containing a lower amount of precoated fibronectin [Fig. 5G]. The inhibition of ROCK activity caused a decrease in the association of PTEN and ROCK [Fig. 5H]. These results clearly show that activation of the RhoA-ROCK-PTEN signaling pathway is strongly upregulated with the decreasing strength of cell adhesion to substrates. Furthermore, the downregulation of the activity of PTEN, a downstream effector of RhoA-ROCK, upregulated the activity of AKT. The reduction of PTEN levels by

siRNA upregulated the phosphorylation of AKT, which was also downregulated in the cells cultured on the HPB substrates [Fig. 5I].

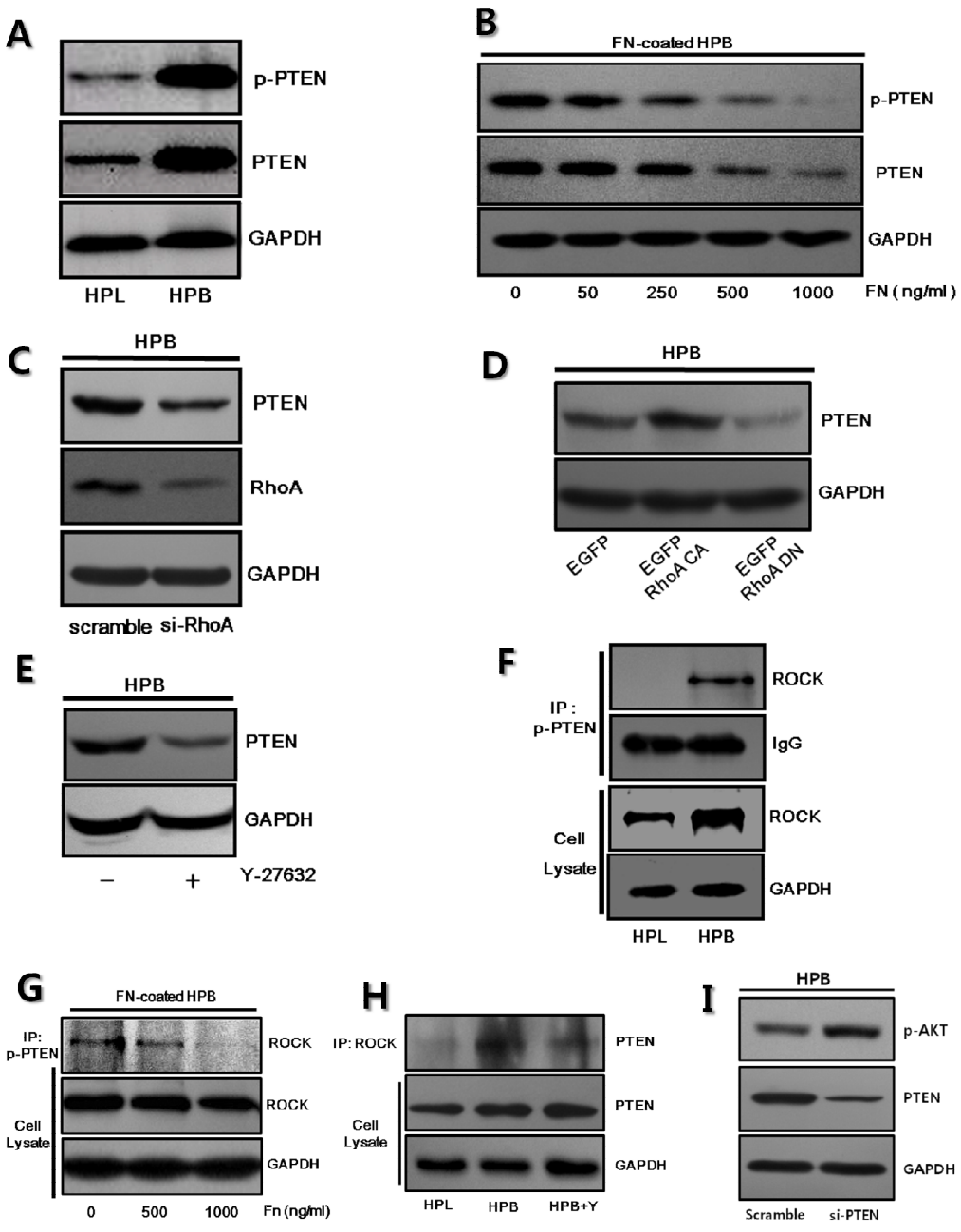


Fig. 5 Downregulation of PTEN activity, which is upregulated by activated

RhoA-ROCK, activates Akt in MC3T3-E1 cells grown on the HPB substrates.

(A) The cells were cultured on the HPL or HPB substrates for two days. The cell lysate of each group was immunoblotted to assess expression or phosphorylation levels of PTEN using anti-p-PTEN or anti-PTEN antibodies. (B) The cells were cultured for two days on HPB substrates that had been precoated with various amounts of fibronectin (FN). The cell lysate of each group was immunoblotted to assess the expression or phosphorylation levels of PTEN using anti-p-PTEN or anti-PTEN antibodies. (C) The cells were cultured on the HPB substrates and transfected with control siRNA (scramble) or with siRNA targeting RhoA (si-RhoA) for 48 h. The cell lysate of each group was immunoblotted to assess expression levels of PTEN and RhoA using anti-PTEN or anti-RhoA antibodies, respectively. (D) The cells were transiently transfected with pCDNA3-EGFP (transfection control), pCDNA3-EGFP-RhoA T19N (dominant negative RhoA), or pCDNA3-EGFP-RhoA

Q63L (constitutively active RhoA) and further cultured for 24 h. Then, the cell lysate of each group was immunoblotted to assess expression levels of PTEN using the anti-PTEN antibody. (E) The cells grown on the HPB substrates were incubated with or without Y-27632 (10 μM) for 24 h. Then, the cell lysate of each group was immunoblotted to assess expression levels of PTEN using the anti-PTEN antibody. (F) The cells were grown on the HPL or HPB substrates for 24 h. Then, the cell lysate of each group was analyzed by immunoprecipitation methods using anti-p-PTEN and anti-ROCK1 antibodies. (G) The cells were grown for 24 h on HPB substrates that had been precoated with various amounts of fibronectin. Then, the cell lysate of each group was analyzed by immunoprecipitation methods using anti-p-PTEN and anti-ROCK1 antibodies. (H) The cells were grown on the HPL or HPB substrates for 5 h. The cells grown on the HPB substrates were incubated with or without Y-27632 (10 μM) for 3 h. Then, the cell lysate of each group was analyzed by immunoprecipitation methods using anti-PTEN or anti-

ROCK1 antibodies. GAPDH levels were monitored for equal loading of samples. (I) Akt phosphorylation was assessed in the cells grown on the HPB substrates for 48 h after transfection with control siRNA (scramble) or with siRNA targeting PTEN (si-PTEN).

1.4 Crosstalk between the RhoA-ROCK-PTEN pathway and phosphatidylinositol 3-kinase in regulating cell proliferation

Next, it was examined if the regulation of AKT phosphorylation by the RhoA-ROCK-PTEN pathway is dependent on PI3K. It is well-established that PTEN antagonizes the effects of phosphatidylinositol 3-kinase (PI3K) to phosphorylate AKT by dephosphorylating PIP3 to create PIP2 [38, 46, 47]. In this study, the cells were treated with inhibitors of PI3K (LY-294002), RhoA (C3), ROCK (Y-27632), or PTEN (bpV(Phen)) and confirmed that the RhoA-ROCK-PTEN pathway downregulates AKT phosphorylation by antagonizing the effect of the growth factor-stimulated activation of PI3K [Fig. 6A-C]. Thus inhibiting the RhoA-ROCK-PTEN pathway reversed the downregulation of cell proliferation by inhibition of PI3K [Fig. 6D-F].

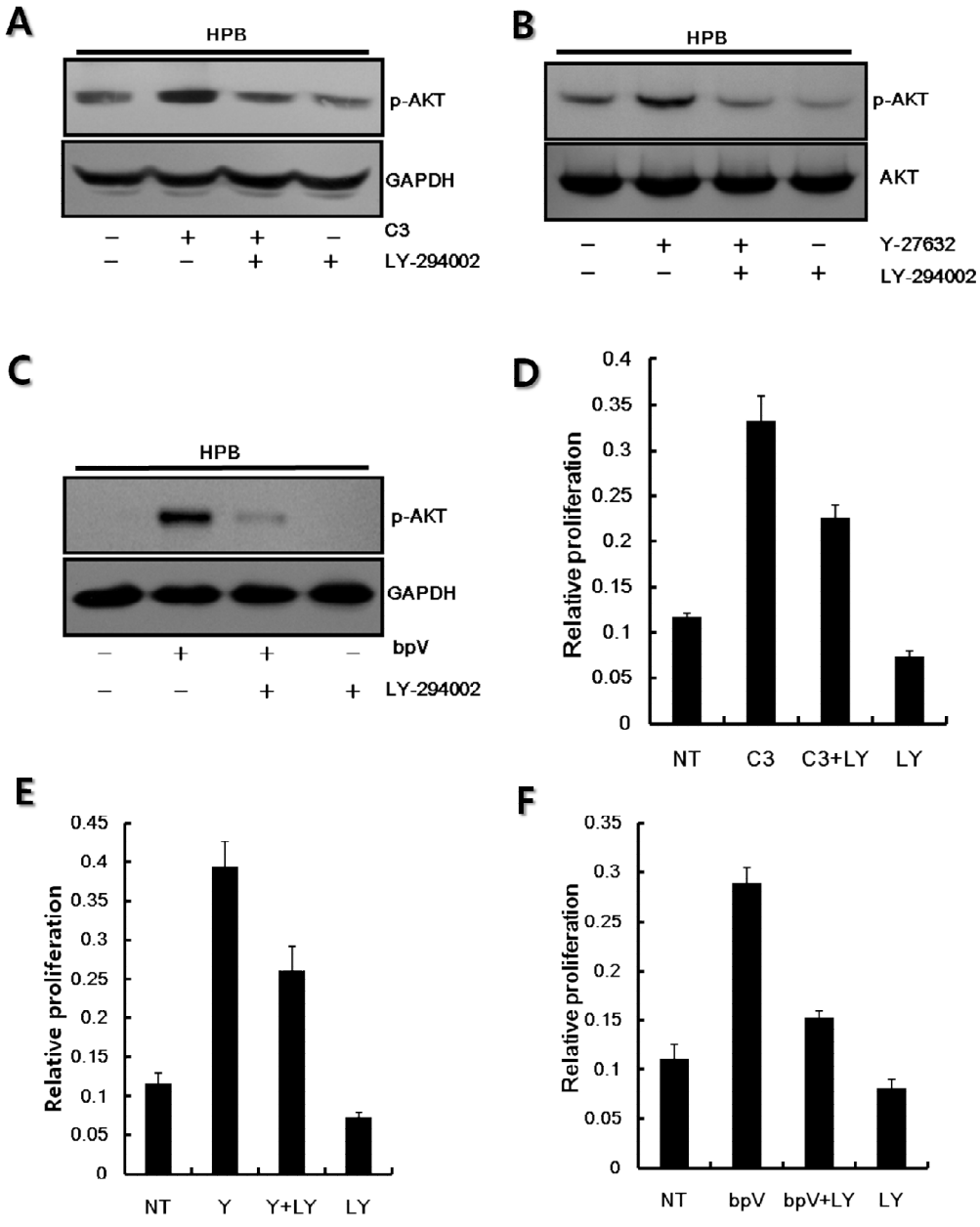


Fig. 6 Inhibition of RhoA, ROCK, or PTEN upregulates Akt phosphorylation

and cell proliferation, which are reversed by PI3K inhibition in MC3T3-E1 cells grown on the HPB substrates.

(A – C) The cells were cultured for two days on the HPB substrates. The cells were then treated with C3 (200 ng/ml) to inhibit RhoA, Y-27632 (10 μM) to inhibit ROCK, or bpV(Phen) (5 μM) to inhibit PTEN in the presence or absence of LY-294002 (10 μM) to inhibit PI3K. The cells were treated with each specific inhibitor for 3 h. Then, the cell lysate of each group was immunoblotted to assess the phosphorylation levels of Akt using the anti-p-Akt antibody. Growth factor receptor signaling was activated by treating cells with FGF2 (50 ng/ml) to activate Akt for 5 min before the cell lysate was prepared. GAPDH levels were monitored for equal loading of cell lysate. (D – F) The cells were cultured for three days on the HPB substrates. Then, the cells were treated with C3 (200 ng/ml) to inhibit RhoA (D), Y-27632 (10 μM) to inhibit ROCK (E), bpV(Phen) (1 μM) to inhibit PTEN (F) in the presence or absence of LY-294002 (10 μM) to inhibit PI3K. The cells were

treated with each specific inhibitor for three days. Then, the relative cell proliferation rates were determined by assessing the cell number with the cell counting kit (CCK-8). NT, Y, or LY indicate the non-treated, Y-27632-treated, and LY-294002-treated groups, respectively. The data are expressed as the mean ± SD. n = 3 dishes. The p-value was less than 0.05 for comparisons between any two groups.

1.5 The role of FAK in the anchorage dependence of cell growth

It has been suggested that FAK is associated with the progression of the cell cycle in anchorage dependent cells[48]. Anchorage-independent growth of cancer cells is related to the activation or overexpression of FAK [19, 49, 50]. In this study, it was found that the phosphorylation of FAK at Tyr397 was lower in the cells grown on the HPB substrates than in the cells grown on the HPL substrates [Fig. 1C]. Therefore, it was investigated that the involvement of FAK in the molecular switch for anchorage dependence. Cell adhesion signaling was blocked by the treatment of cells with FAK inhibitor 14 or siRNA knockdown of FAK in cells grown on the hydrophilic tissue culture plates, where cell proliferation was normal. The inhibition of FAK with FAK inhibitor 14 or siRNA caused the cells to detach from the substrate or delayed cell proliferation, whereas ROCK inhibition rescued this activity [Fig. 7A]. FAK inhibition increased phosphorylation of MLC indicating increased ROCK activity [Fig. 7B] as well as PTEN activity, which was reversed

by the inhibition of ROCK [Fig. 7C]. In addition, FAK inhibition decreased AKT phosphorylation, but, consistent with the assumption, the inhibition of RhoA (by C3), ROCK (by Y-27632), or PTEN (by bpV(Phen)) rescued the phosphorylation of AKT [Fig. 7D]. ROCK inhibition also reversed the slow cell proliferation rate caused by FAK inhibition [Fig. 7E]. Thus, all of these data indicate that low FAK activity fails to suppress the activity of the RhoA-ROCK-PTEN pathway in cells that are weakly attached to substrates.

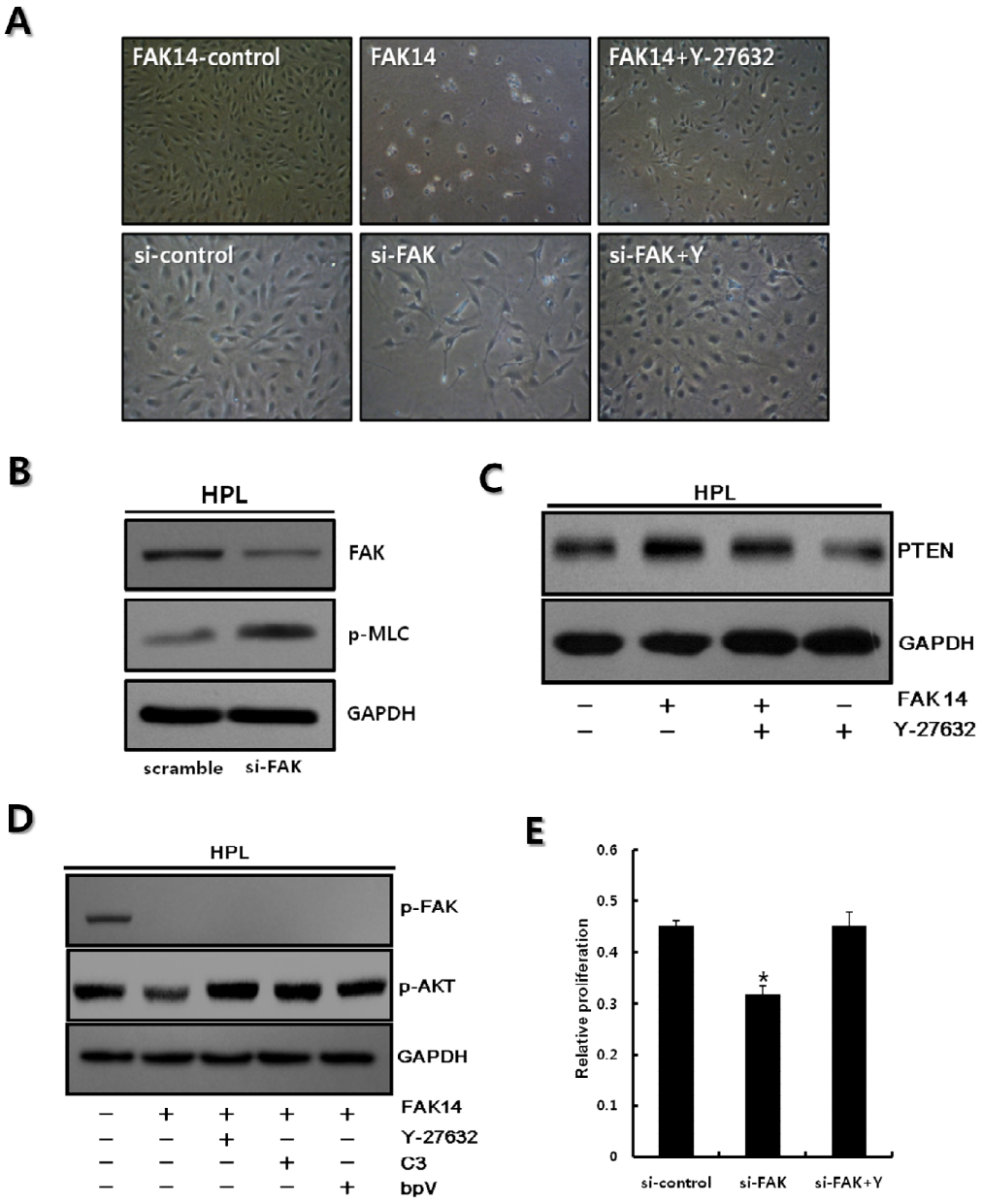


Fig. 7 The low activity of MC3T3-E1 cells induced by FAK inhibition is

reversed by inhibiting the activity of RhoA-ROCK-PTEN on the HPL substrates.

(A) The cells were cultured on the HPL substrates for three days in the presence of FAK inhibitor 14 (3 μM) to inhibit FAK with or without Y-27632 (20 μM) to inhibit ROCK. In addition, the cells on the HPL substrates were transfected with siRNA targeting FAK. After 18 h of transfection, the cells were treated with Y-27632 (10 μM) to inhibit ROCK. Then, cells were further cultured for three days before observation by phase contrast microscopy. (B) MLC phosphorylation of cells grown on the HPL substrates for 72 h after transfection with control siRNA (scramble) or with siRNA targeting FAK (si-FAK) was assessed. (C) The cells were cultured with FAK inhibitor 14 (1 μM) or Y-27632 (10 μM) on the HPL substrates for 24 h. The cell lysate of each group was immunoblotted to assess the expression level of PTEN using the anti-PTEN antibody. (D) The cells grown on HPL substrates were treated with various combinations of FAK inhibitor 14 (1 μM), Y-

27632 (10 μM), C3 (200 ng/ml) or bpV(Phen) (5 μM) for 3 h as indicated in the figure. The cell lysate of each group was immunoblotted to assess the phosphorylation levels of Akt and FAK using the anti-p-Akt and anti-p-FAK antibodies, respectively. Growth factor receptor signaling was activated by treating cells with FGF2 (50 ng/ml) to activate Akt for 5 min before harvest of the cell lysate. (B - D) GAPDH levels were monitored for equal loading of cell lysate. (E) The cells on the HPL substrates were transfected with siRNA targeting FAK. After 18 h of transfection, the cells were treated with Y-27632 (20 μM) to inhibit ROCK. The cells in the control groups were not treated with Y-27632. The cells were further cultured for three days. Relative cell numbers were determined with the cell counting kit (CCK-8). The data are expressed as the mean ± SD. n = 3 culture dishes. *, $P < 0.05$ when compared to si-control or si- FAK + Y samples.

2. Cells in suspension

2.1 Culture of cells in suspension

The regulation of ROCK and PTEN activities by cell adhesion was further confirmed in NIH 3T3 fibroblasts, which were cultured in the absence of cell adhesion on poly-HEMA substrates. More ROCK-PTEN complex was formed in the fibroblasts cultured in suspension on the poly-HEMA substrates than in the cells grown on the tissue culture dishes [Fig. 8A]. The inhibition of ROCK decreased the activity of PTEN, which was demonstrated by a decrease in the amount of PTEN associated with ROCK [Fig. 8A] or the amount of phosphorylated PTEN [Fig. 8B]. AKT phosphorylation was also increased by ROCK inhibition in the cells cultured in suspension on poly-HEMA substrates [Fig. 8B]. The knockdown of RhoA with siRNA decreased the phosphorylation of PTEN and upregulated AKT phosphorylation [Fig. 8C]. These results further indicate that the RhoA-ROCK-PTEN pathway is a molecular switch for anchorage dependence

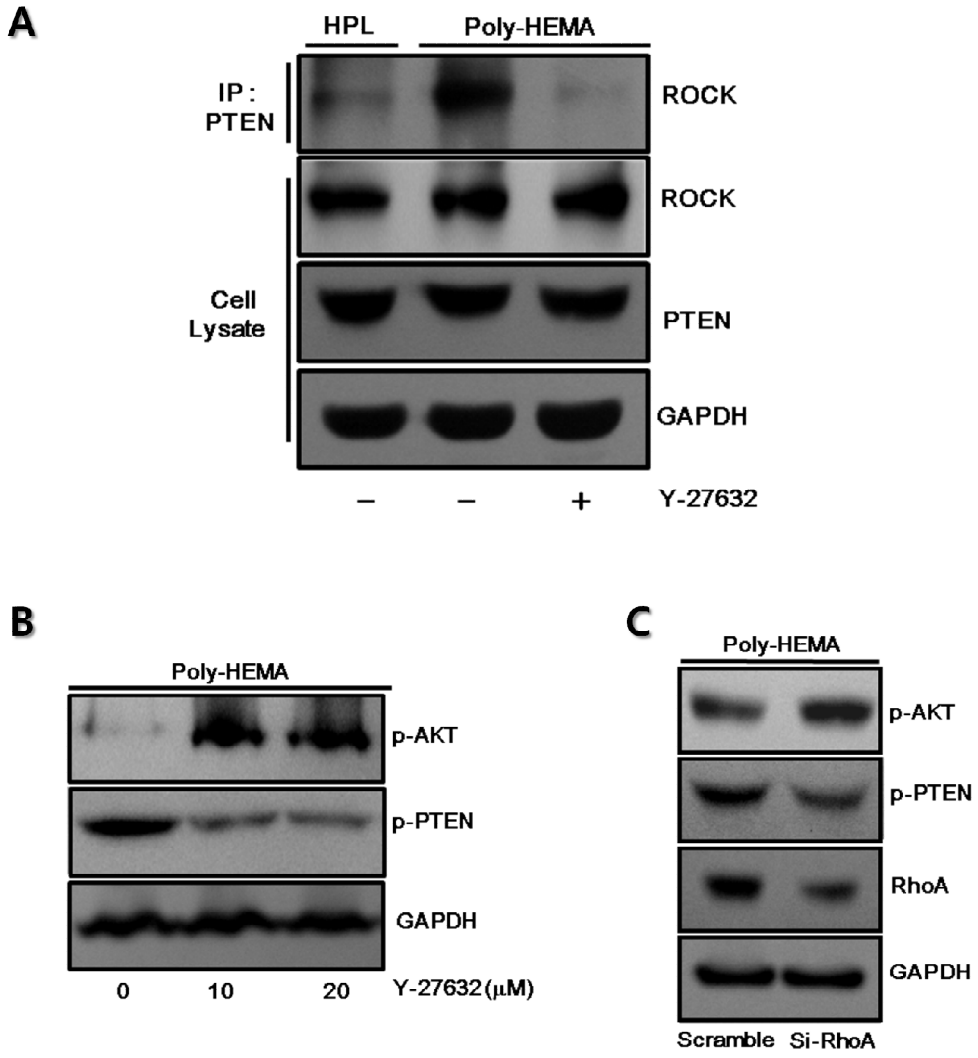


Fig. 8 The role of RhoA–ROCK–PTEN in anchorage dependence was confirmed using NIH 3T3 fibroblasts grown over poly–HEMA substrates.

(A) Fibroblast cells were replated onto HPL substrates or poly–HEMA

substrates to abolish cell adhesion and cultured for 3 h. Cells plated onto poly-HEMA substrates were incubated in the absence or presence of Y-27632 (10 μM). Then, the cell lysate of each group was analyzed by immunoprecipitation methods using anti-PTEN or anti-ROCK1 antibodies. The levels of GAPDH were monitored for equal loading of samples. (B) Fibroblasts grown on the poly-HEMA substrates were incubated with or without Y-27632 (10 or 20 μM) for 3 h. The cell lysate of each group was immunoblotted to assess the phosphorylation levels of AKT and PTEN using anti-p-AKT or anti-p-PTEN antibodies. (C) Fibroblasts grown on the HPL substrates were transfected with control siRNA (scramble) or siRNA targeting RhoA (si-RhoA) and further cultured for 48 h. Then, the cells were replated onto poly-HEMA substrates and incubated for 5 h. The cell lysate of each group was immunoblotted to assess phosphorylation levels of AKT and PTEN using anti-p-AKT or anti-p-PTEN antibodies, respectively. (B and C) Growth factor receptor signaling was activated by treating the cells with

FGF2 (50 ng/ml) to activate Akt for 5 min before harvest of the cell lysates. GAPDH levels were monitored for equal loading of samples.

2.2 The effect of ROCK inhibition in weak adhesion signal-induced cell apoptosis.

Weak cell adhesion signal induced by the HPB and the Poly-HEMA surface activated apoptotic pathway such as caspase-3 and finally induced cell death. ROCK inhibition by Y-27632 decreased cell death on the HPB surface for long-term culture (Fig. 9A). The inhibition of RhoA-ROCK-PTEN signal pathway also downregulated caspase-3 activity, which is the most critical molecule in apoptotic progress, induced by the HPB surface or Poly-HEMA (Fig. 9B and C).

Next, the effect of ROCK inhibition on the cell viability was measured under weak adhesion-induced apoptotic conditions such the HPB surface and Poly-HEMA surface. ROCK inhibition improved cell viability for suspension condition (Fig. 9D and E). Particularly, the cell viability was dramatically increased by ROCK inhibition under long-term culture with anoikis stress (Fig. 9F and G).

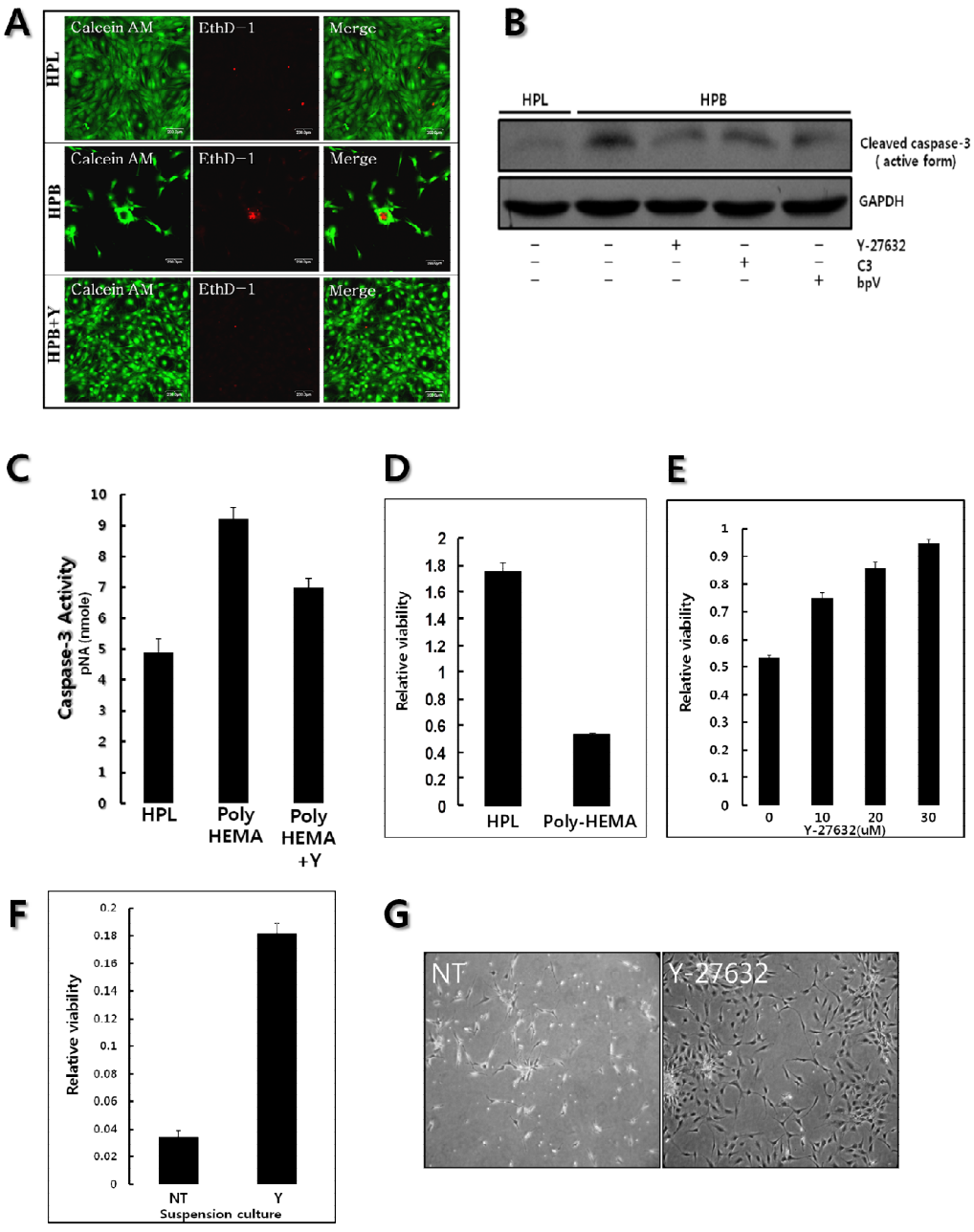


Fig . 9 ROCK inhibition by Y-27632 inhibits weak cell adhesion signal-induced cell apoptosis.

(A) MC3T3-E1 cells were grown with or without Y-27632 (20 μM) on the HPB surface for 5 days. The cells grown on the HPL surface were used as control. Cell viability was analyzed by Live/Dead assay. Green color and red color indicate live cells and dead cells respectively. (B) The cells were incubated with y-27632 (20 μM), C3 (200 ng/ml) or bpV (1 μM) on the HPB surface for 3days. Active caspase-3 was measured by immunoblotting using anti-cleaved caspase-3 antibody. (C) The cells were incubated with or without Y-27632(20 μM) on Poly-HEMA surface for suspension culture for 24hr. Caspase-3 activity was assessed using ApoAlert caspase-3 activity assay kit. (D and E) NIH3T3 cells were incubated with or without Y-27632 for 24h on the Poly-HEMA surface. Cell viability assay was performed by CCK-8 kit. (F and G) MC3T3-E1 cells were cultured for 1 week upon suspension condition with or without Y-27632. And then, the cells were replated on the HPL surface. CCK-8 kit was used for measuring cell viability.

2.3 Reattachment of cells to substrates

The dependence of the activation of the RhoA-ROCK-PTEN pathway on the strength of cell adhesion was further confirmed by reattaching the detached cells to the substrates. The high RhoA activity of the detached cells decreased upon reattachment to the substrate [Fig. 10A]. However, RhoA activity remained higher in the cells plated onto the HPB substrates than those seeded onto the HPL substrates [Fig. 10B]. The activities of ROCK and PTEN were also higher in the cells plated onto the HPB substrates, where FAK and paxillin were phosphorylated to lower extents than in the cells seeded onto the HPL substrates [Fig. 10C]. The pretreatment of the detached cells with the FAK inhibitor failed to lower ROCK and PTEN activities upon reattachment to the HPL substrates [Fig. 10D]. Thus, these results indicate that FAK-mediated adhesion signaling is too low to downregulate the activity of the RhoA-ROCK-PTEN pathway in cells that are weakly attached to the substrates.

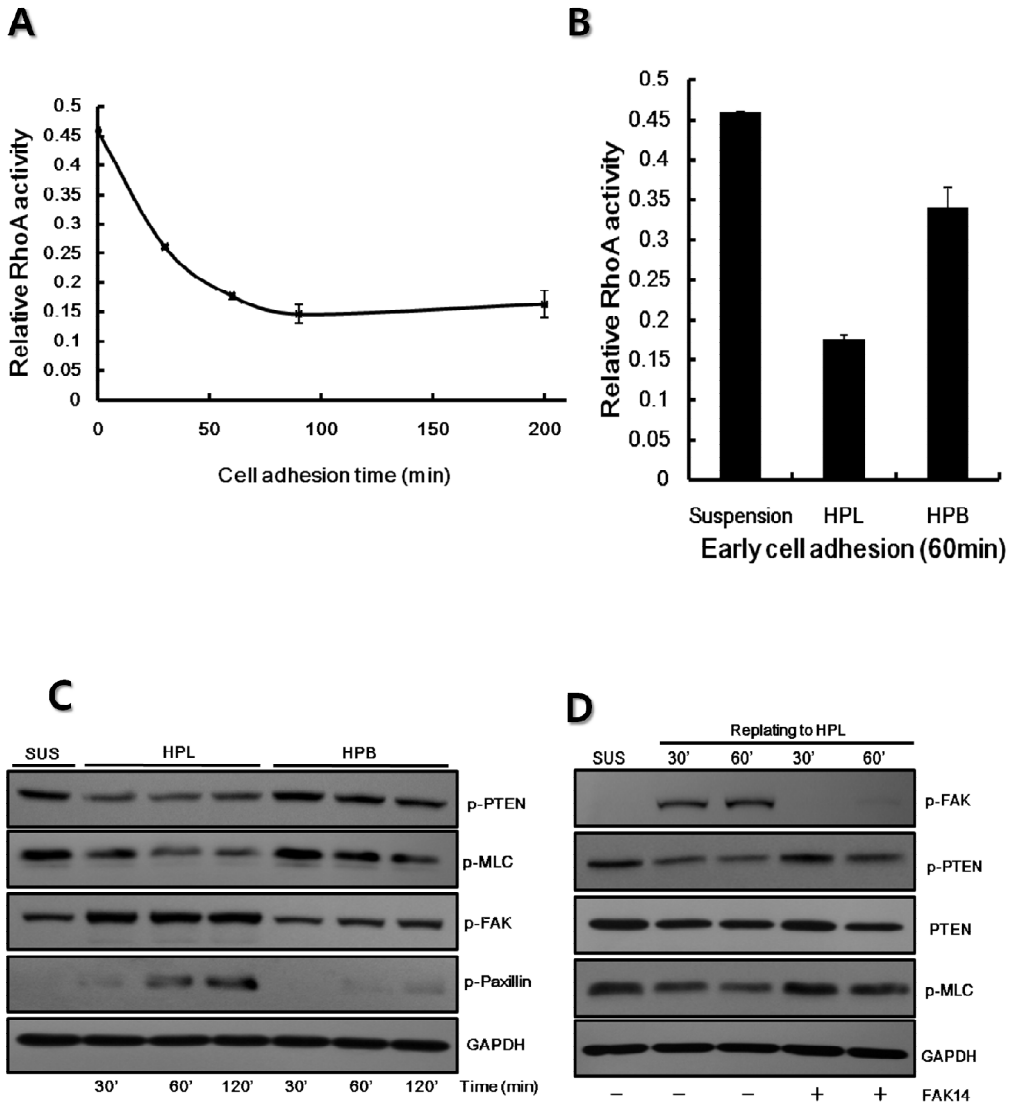
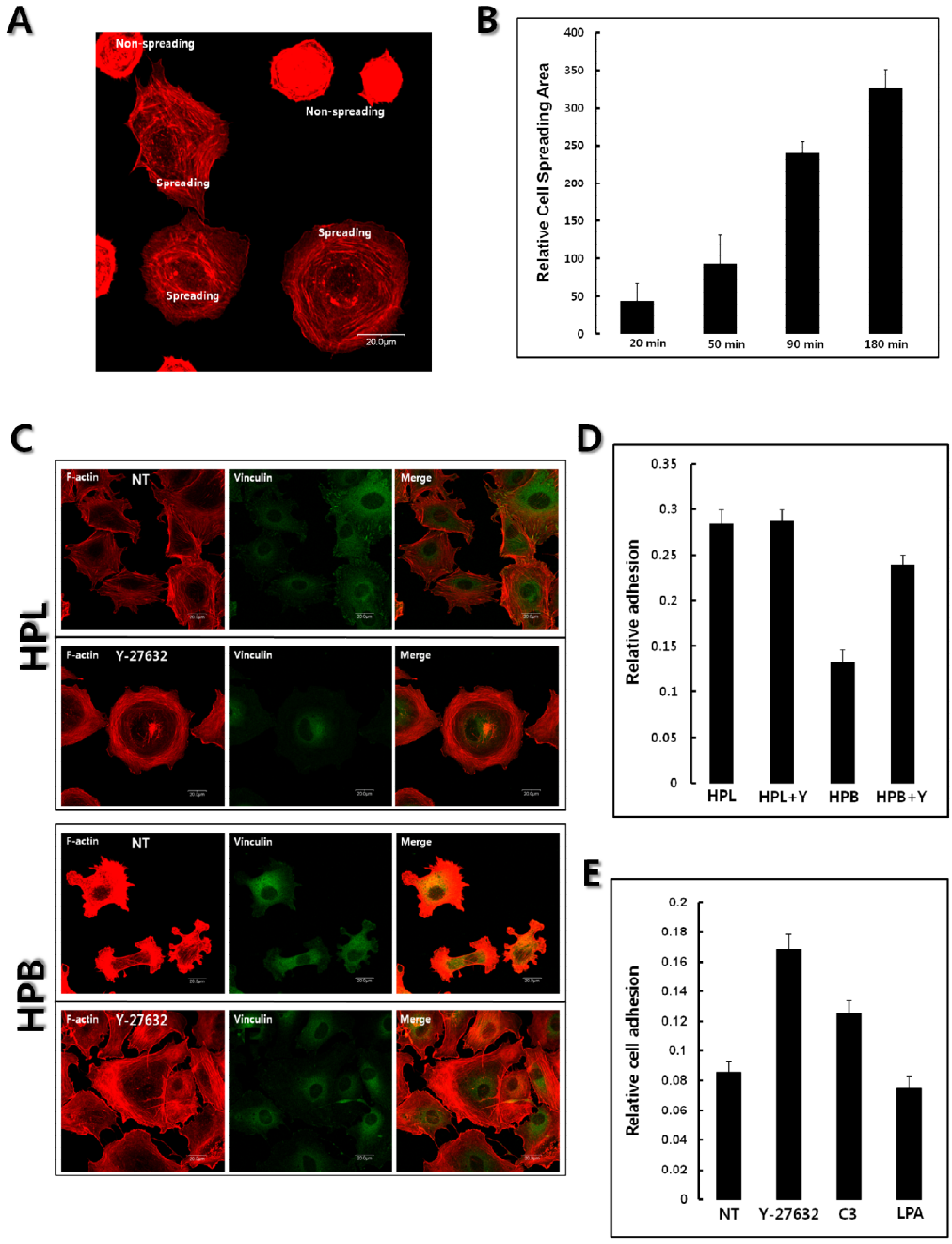


Fig. 10 Detached cells that are reattached to the substrate demonstrate changes in the activities of RhoA, ROCK, and PTEN during cell adhesion. (A) Cells replated onto the HPL substrates were harvested at the

indicated time points up to 3 h after the cells had been seeded. Then, equal amounts of cell lysates were used for the G-LISA RhoA activation assay. The data are expressed as the mean ± SD. n = 3 dishes. (B) The cells were replated onto the HPL or HPB substrates and harvested after 1 h. Equal amounts of cell lysates were used for the G-LISA RhoA activation assay. The data are expressed as the mean ± SD. n = 3 dishes. The p-value was less than 0.05 for comparisons between any two groups. (C) The cells were replated onto the HPL or HPB substrates and then harvested at the indicated time points. The cell lysate of each group was immunoblotted to assess the phosphorylation levels of PTEN, MLC, FAK, and paxillin using anti-p-PTEN, anti-p-MLC, anti-p-FAK and anti-p-paxillin antibodies, respectively. (D) The cells were replated onto the HPL substrates in the absence or presence of FAK inhibitor 14 (1 μM) to inhibit FAK and then harvested at the indicated time points. Cell lysates of each group were immunoblotted to assess the phosphorylation levels of FAK, PTEN and MLC using anti-p-FAK, anti-

p-PTEN, anti-PTEN and anti-p-MLC antibodies, respectively. (C – D) GAPDH levels were monitored for equal loading of cell lysate.

Next, spreading of cell was further examined during the early cell attachment. Cell spreading was accompanied with loosening circular F-actin bundles which compactly filled the small and globular cells seeded to the substrates [Fig. 11A and B]. Cell attachment to the HPB substrates was improved by inhibiting RhoA or ROCK activity [Fig. 11C - E]. The cells transfected with constitutively active RhoA did not spread up to 24 h of culture on the HPL substrates [Fig. 11F and G]. ROCK activity remained high in the cells seeded to the HPB surface, whereas it decreased in the cells plated to the HPL surface [Fig. 11H]. These data indicate that cell spreading during the cell attachment to the substrates accompanies with unwinding F-actin bundles which have been tightly packed upon cell detachment from the substrates by RhoA-ROCK activation. Therefore, downregulation of RhoA-ROCK activity by adhesion signal may be required for cells to spread on the substrates during the early cell attachment.


A
Non-spreading
Non-spreading
Spreading
Spreading
Spreading
20.0μm
B
Relative Cell Spreading Area
400
350
300
250
200
150
100
50
0
20 min
50 min
90 min
180 min
C
HPL
F-actin
NT
Vinculin
Merge
F-actin
Y-27632
Vinculin
Merge
HPB
F-actin
NT
Vinculin
Merge
F-actin
Y-27632
Vinculin
Merge
D
Relative adhesion
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HPL
HPL+Y
HPB
HPB+Y
E
Relative cell adhesion
0.2
0.18
0.16
0.14
0.12
0.1
0.08
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NT
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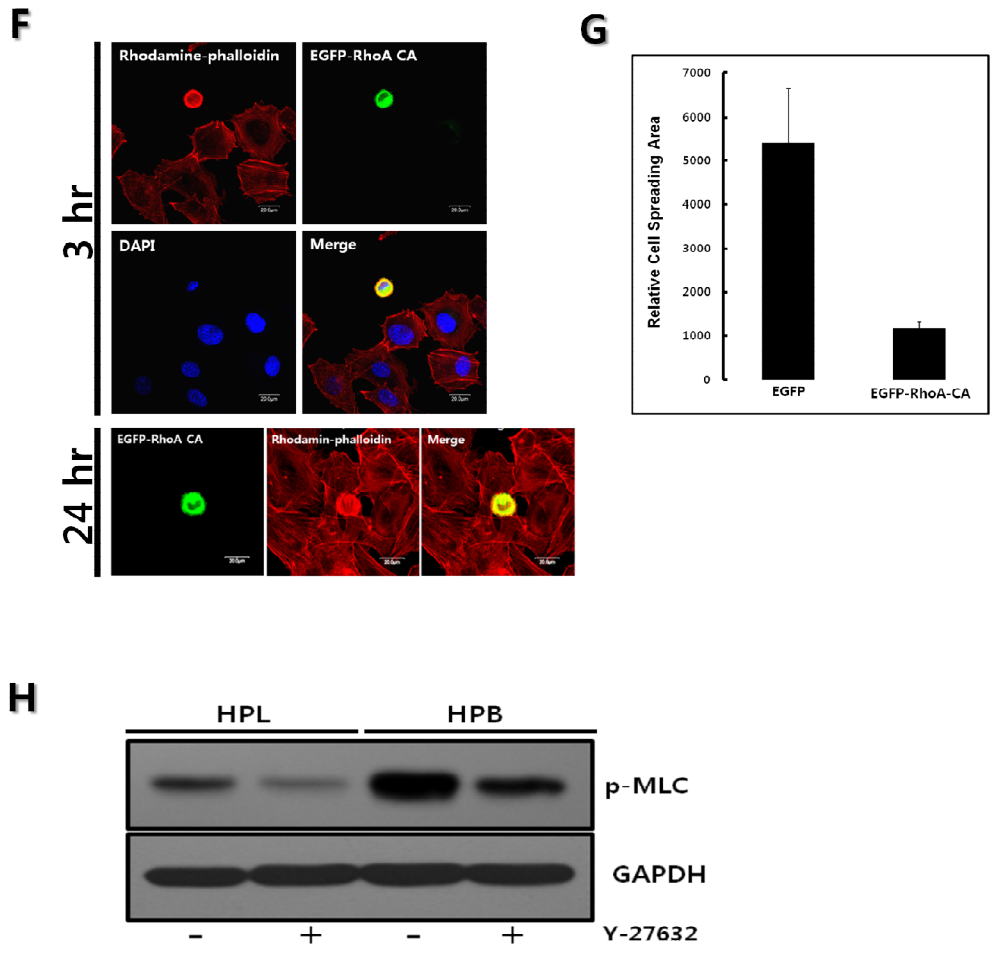


Fig 11. Downregulation of RhoA-ROCK activity is essential for cell to spread during the early cell adhesion to the susbtrates. (A) Immunohistochemical observation of MC3T3-E1 cells which were cultured on the HPL substrates for 1 h. Cells were fixed and stained with

rhodamine-phalloidin for F-actin. (B) The spreading area of cells during the initial attachment were measured using Image J program. (C) Cells were plated with or without Y-27632 treatment on HPL or HPB. After 3h culture, the cells were fixed and stained with rhodamine-phalloidin for F-actin. (C) Cells were plated with or without Y-27632 treatment on HPL or HPB. After 3h culture, cell number was measured by CCK-8 kit. (E) Cell number was measured by CCK-8 kit after 6 h culture of cells on the HPB surface with Y-27632, C3 or LPA treatment. (F) The cells transfected with pCDNA3-EGFP-RhoA Q63L (constitutively active RhoA) were replated onto the HPL substrates for 24h. Then the cells were fixed and stained with rhodamine-phalloidin for F-actin. The green color indicates the cell which expresses constitutively active RhoA. (F) To determine the spreading area of MC3T3-E1 cells expressing EGFP-RhoA- Q63L during the early cell adhesion, the cell sizes were measured by Image J program for the cells cultured for 3h. (H) Cells were seeded onto the HPL or HPB surface with or without Y-27632

treatment. After 24h culture, the level of p-MLC was assessed by immunoblotting to determine ROCK activity indirectly.

3. Cells on PCA Substrates

3.1 Proliferation and spreading of osteoblasts

MC3T3-E1 cell proliferation rate was slow on the calcium phosphate apatite (PCA) surface. After 3 days of culture, the cell number on the PCA surface was about 30% of that grown on regular tissue culture plastic HPL surface [Fig. 12B]. Microscopic examination of the cultured cells showed that osteoblasts spread less on the PCA surface. The cells had characteristically less cytoplasm around the nucleus and extended several slender and long cell processes on the PCA surface compared to the cells cultured on the HPL surface [Fig. 12C]. Extent of cell spreading is known to be dependent on the developments of stress fibers and focal adhesions[24-27, 51]. In the previous studies, actin filaments in the stress fibers were stained with phalloidin-FITC and focal adhesions were stained with anti-vinculin antibody to examine the possibility that the low cell proliferation rate on PCA surface may be due to poorly developed stress fibers and focal adhesions. Many stress fibers that link

focal adhesions were clearly visible in cells grown on the HPL surface, but in cells grown on PCA surface only diffuse staining of cytoplasm by phalloidin occurred without characteristic actin strands, indicating that stress fibers and focal adhesions did not develop well on the PCA surface .

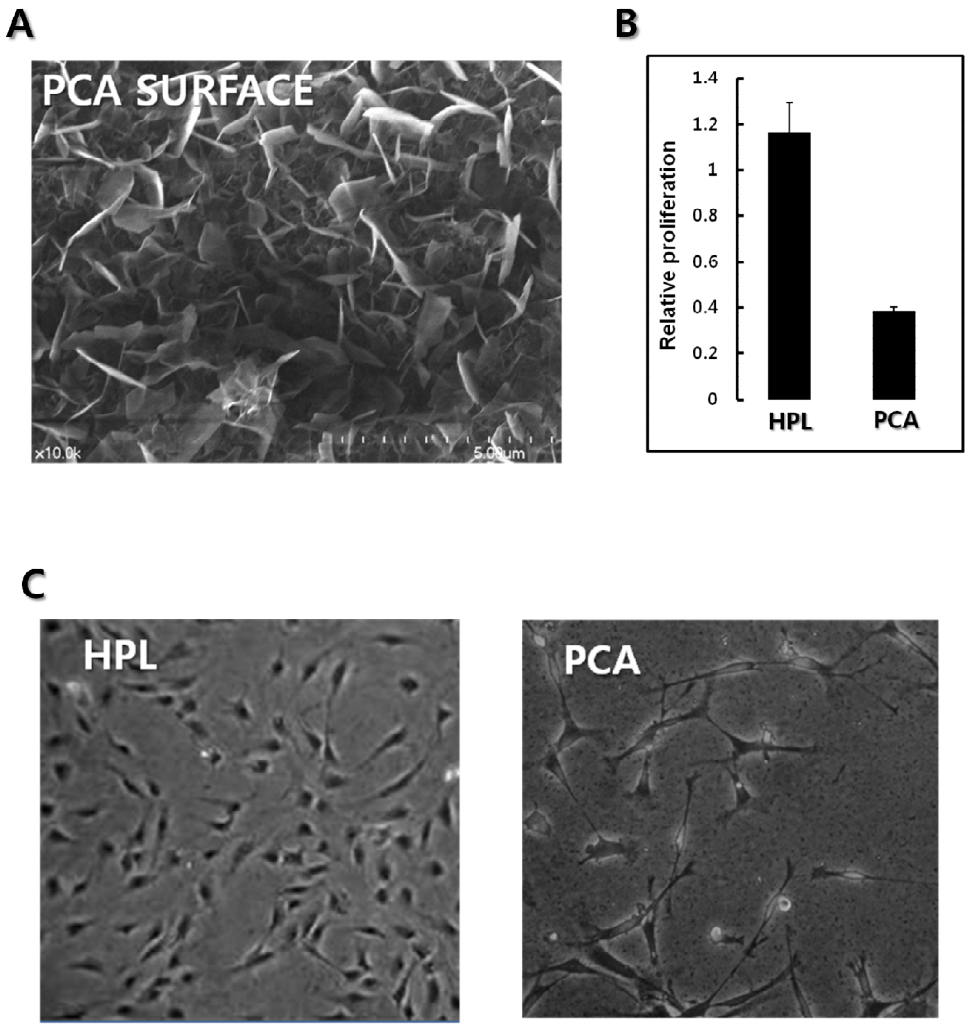


Fig. 12 Proliferation of osteoblasts is slow and the cell spreading is poor on the PCA surface.

(A) FE-SEM view of the PCA surface. x 10,000. (B) Proliferation of

osteoblasts determined by CCK-8 method and expressed as the relative number of MC3T3-E1 cells cultured for three days on the HPL (tissue culture plate) or the PCA (poorly crystalline calcium phosphate apatite) surfaces. Data expressed as mean ± SD. n=3 culture dishes. *, P< 0.05 (C) Phase contrast microscopy of osteoblasts cultured for one day on HPL or PCA.

3.2 Effects of ROCK inhibition on cell proliferation and spreading

It have previously been reported that inhibition of ROCK markedly enhances adhesion and proliferation of osteoblasts grown on hydrophobic surfaces where adhesion signaling of osteoblasts is weakly generated [14] similar to the cells grown on PCA thin film in the present study. Therefore, further study was performed by introducing previous aspects. The effects of ROCK inhibition were tested on osteoblast proliferation and the cell morphologic changes. Treatment with 10 μM Y-27632 for a specific inhibitor of ROCK, C3 for Rho inhibition or LPA for Rho activation for 3 days in osteoblasts cultured on the PCA surface markedly enhanced the proliferation rate and induced cell spreading except for the cell treated with LPA [Fig. 13 A-C].

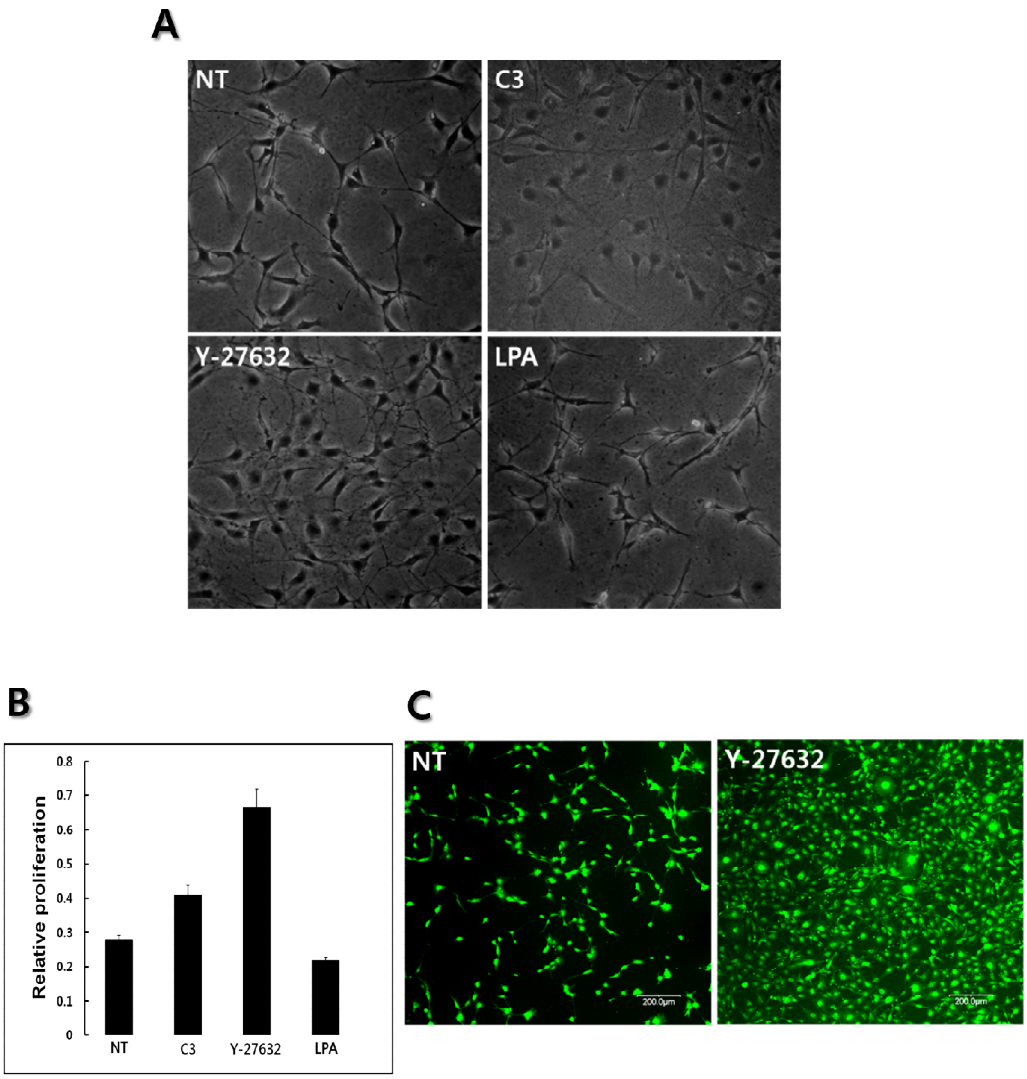


Fig. 13 Rho and ROCK inhibition upregulates cell proliferation in MC3T3-E1 osteoblasts grown on the PCA surface. (A) MC3T3-E1 cells were

incubated with C3 (0.1 μg/ml), Y-27632 (10 μM) or LPA (10 μM). After 24h, the cells were observed. (B) Cell proliferation on PCA was enhanced in the presence of 10 μM of Y-27632 and 0.1 μg/ml of C3 during 3 days of culture. Data expressed as mean ± SD. n=3 culture dishes. *, $P<0.05$ compared to respective untreated controls. (C) The cells were incubated with or without Y-27632 on the PCA and Calcein AM was added for live cell. Cell morphology on PCA were observed by phase contrast microscopy and fluorescent Microscopy

3.3 Effects of ROCK inhibition on Akt

To investigate the molecular mechanism of ROCK inhibition in cell proliferation, the effects of ROCK inhibitor Y-27362 was determined on the phosphorylation of Akt, a key protein kinase that controls cell-cycle progression[52]. Increased proliferation of osteoblasts grown on PCA surface following ROCK inhibition increased the level of phosphorylated-Akt [Fig. 14A and B]. Akt is a downstream effector of phosphatidylinositol 3-kinase (PI3K) that increases cellular concentration of phosphatidylinositol-3,4,5-triphosphate (PIP3), binding of which to the pleckstrin homology domain of Akt membrane localizes the enzyme for phosphorylation-dependent activation [38, 46, 53]. Pharmacologic inhibition of PI3K is expected to block activation of Akt. Indeed, treatment with LY-294002, a specific inhibitor of PI3K, largely blocked the Y-27632-dependent proliferation in osteoblasts and markedly reduced the phosphorylation of Akt [Fig. 14A and B]. Together with data presented above, the results of this experiment indicate that

osteoblasts grown on PCA surface generate weak adhesion signaling and have reduced cell proliferation and downregualted Akt phosphorylation which limits cells to progress into the cell cycle.

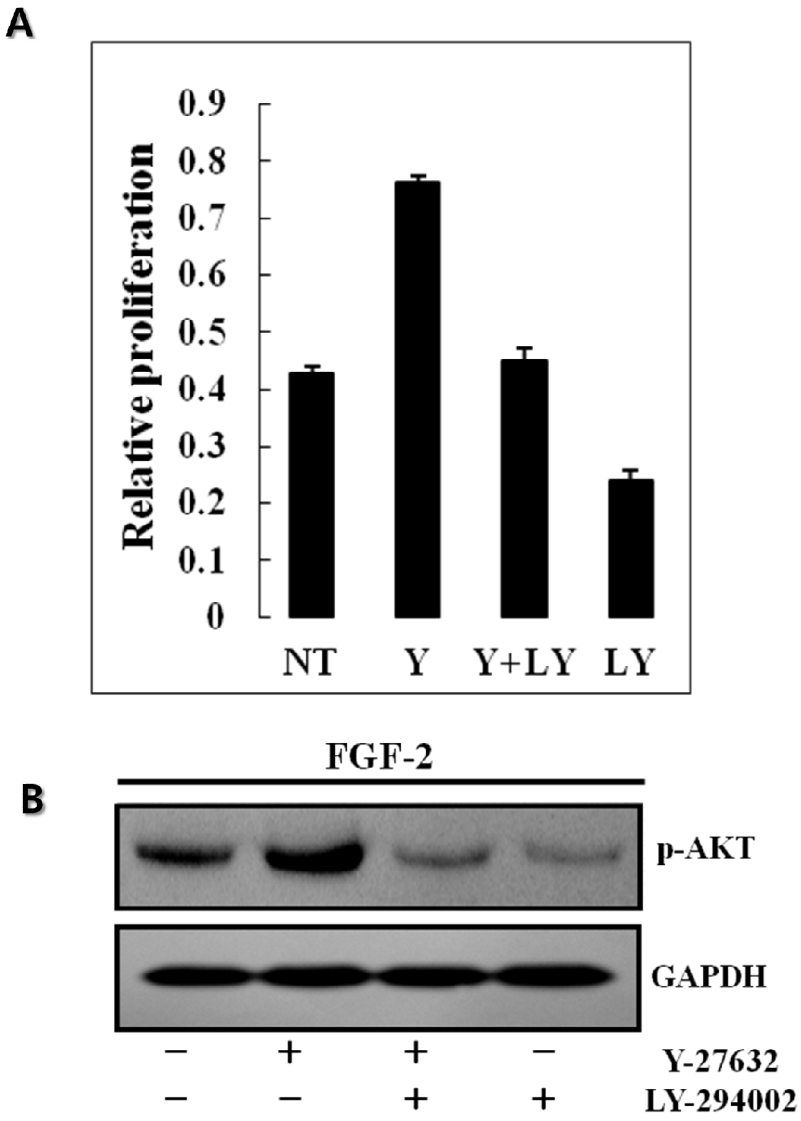


Fig. 14 ROCK inhibition of MC3T3-E1 osteoblasts increases cell proliferation, Akt activity on the PCA surface.

(A) Effects of ROCK and PI3K inhibitions on cell proliferation of

osteoblasts on PCA. Relative cell proliferation was determined in the presence and absence of 10 μM Y-27632 (ROCK inhibitor, Y) and 10 μM LY-294002 (PI3K inhibitor, LY). Data expressed as mean ± SD. n=3 culture dishes. The p-value was less than 0.05 between comparison of any two groups except for NT and Y+LY. NT: non-treated control. (B) Effect of ROCK inhibition and PI3K inhibition on Akt phosphorylation in osteoblasts on PCA. Three hours prior to harvest of cells grown for 24 hr on PCA surface, osteoblasts were treated with and without Y-27632 (10 μM) and/or LY-294002 (10 μM). Growth factor receptor was activated with FGF2 (50 ng/ml) for 5 min before harvest and immunoblotted for Akt.

3.4 Effects of RhoA on cell proliferation

Inhibition of ROCK promoted spreading of osteoblast on PCA surface and cell proliferation. ROCK is involved in a wide range of different cellular processes as a downstream effector protein of the Rho family of GTPases which is one of the major regulators of cytoskeleton and cell adhesion-related structures intracellularly [29, 30, 54, 55]. To confirm signaling upstream of ROCK, RhoA activity was investigated in osteoblasts grown on PCA surface. RhoA signaling was robust in osteoblasts grown on the PCA surface. Total cellular RhoA was elevated more than two-fold in osteoblasts on the PCA surface compared to the cells on the HPL surface where the stress fibers and focal adhesions were well developed [Fig. 15A]. Immunoblotting with anti-Rho antibody also confirmed the striking increase of RhoA levels in osteoblasts grown on the PCA compared to those grown on HPL surface [Fig. 15B]. In addition, Akt phosphorylation was higher in HPL-grown cells than PCA-grown cells, consistent with the idea that cell signaling that control cell

proliferation is downregulated in osteoblasts grown on the PCA surface [Fig. 15C].

To confirm that the increased total cellular RhoA activity downregulates cell proliferation, the osteoblasts were transfected with constitutively active RhoA (EGFP-RhoA Q63L) or dominant negative RhoA (EGFP-RhoA T19N) forms and determined the levels of Akt phosphorylation in the transfectants grown on PCA surface. Overexpression of dominant negative RhoA increased Akt phosphorylation in MC3T3-E1 osteoblasts on the PCA surface, whereas transfection with constitutively active RhoA decreased Akt phosphorylation [Fig. 15D], in agreement with the notion that increased cellular RhoA activity leads to down-regulation of signaling molecules of the cell proliferation-dependent pathway such as Akt. In addition, siRNA knockdown of cellular RhoA levels increased Akt phosphorylation in cells grown on PCA surface (Fig. 15E). Furthermore, pharmacological inactivation of RhoA by preincubation of cells with cell-permeable C3

transferase (200 ng/mL) also increased Akt phosphorylation [Fig. 15F], and importantly increased the rate of cell proliferation when the osteoblasts were co-cultured in the presence of C3 for three days [Fig. 15H]. In contrast, specific activation of RhoA by LPA decreased Akt phosphorylation [Fig. 15G] and marginally decreased the relative proliferation compared to untreated controls group [Fig. 15I] in osteoblasts cultured on PCA surface. These results demonstrate an inverse correlation between RhoA activity and cell proliferation, and these data are consistent with the notion that activated RhoA/ROCK signaling pathway restrains the cell cycle progression of osteoblasts cultured on the PCA surface.

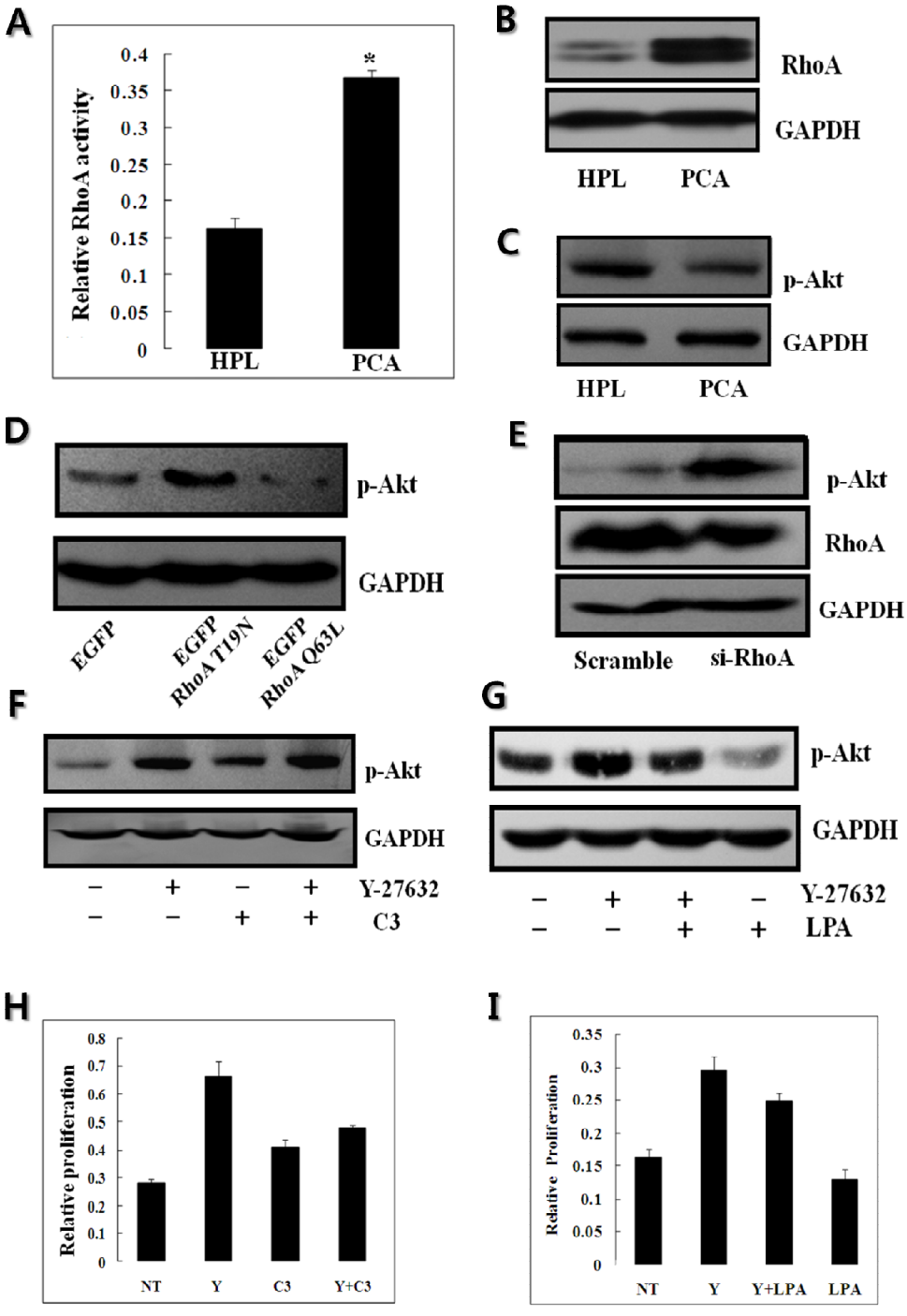


Fig. 15 Akt activity and cell proliferation are associated with high total

cellular RhoA activity on the PCA surface.

(A) Total cellular RhoA activity of MC3T3-E1 osteoblast was determined in cells cultured for two days. Equal amount of cell lysate proteins were used in G-LISA RhoA activation assay. Data expressed as mean ± SD. n=4 culture dishes. *, P< 0.05. (B) RhoA expression is upregulated on PCA compared to HPL surface as determined by immunoblot using anti-RhoA antibody. (C) Akt phosphorylation determined by immunoblotting cells grown on the HPL or PCA surface. (D) Effects of overexpression of dominant negative or constitutively active RhoA on cells grown on the PCA surface. Cells were transiently transfected with pCDNA3-EGFP, pCDNA3-EGFP-RhoA T19N (dominant negative), or pCDNA3-EGFP-RhoA Q63L (constitutively active) and immunoblotted after 24 h. (E) Akt phosphorylation of osteoblasts grown on the PCA surface after transfection with control siRNA (scramble) or with siRNA targeting RhoA (si-RhoA) and immunoblotted after 48 h. Growth factor receptor was stimulated with

FGF-2 (50 ng/ml) to activate Akt for 5 min before cell lysis (C – E). GAPDH was monitored for equal loading of samples. (F and G) Effects of RhoA inactivation by C3 (200 ng/ml) (F) and/or RhoA activation by LPA (10 μM) (G) on Akt phosphorylation. ROCK was inhibited with Y-27632 (10 μM). Phosphorylated Akt was measured by immunoblotting after 3 h treatment. Growth factor receptor was activated by treating cells with FGF2 (50 ng/ml) to activate Akt for 5 min before harvesting cell lysates. (H and I) Effect of RhoA inhibition (H) and RhoA activation (I) on cell proliferation. Cell proliferation determined by CCK-8 method after three days with and without C3 (200 ng/ml), LPA (10 μM) and/or Y-27632 (10 μM). Data expressed as mean ± SD. n = 4 culture dishes. The p-value was less than 0.05 when any two groups were compared within respective experiments (H and I). NT: non-treated

3.5 Effects of PTEN on cell proliferation

Recently, PTEN phosphatase, a tumor suppressor protein was shown to be one of downstream effectors of ROCK [41, 56]. PTEN also inhibits Akt activity by dephosphorylating PIP3 into PIP2[43, 46, 57], counteracting PI3K that activate Akt. The role of PTEN was examined in the regulation of cell proliferation on the PCA surface. Activation level of PTEN was assessed by immunoblotting with anti-PTEN antibody or anti-phosphospecific-PTEN antibody. Phosphorylation prevents degradation of PTEN and is essential for PTEN stability [38, 39]. In this study, both antibodies showed similar results. Total cellular PTEN phosphorylation was clearly enhanced in cells cultured on the PCA surface compared to those cells cultured on the HPL surface [Fig. 16A]. Pharmacological inhibition of PTEN by treatment with bpV(Phen) (bisperoxo(1,10-phenanthroline)oxovanadate) increased Akt phosphorylation in cells cultured on the PCA surface [Fig. 16B]. The increased cell proliferation and Akt phosphorylation following ROCK

inhibition was largely reversed by specifically inhibiting PI3K activity [Fig. 16B] with LY-294002. Furthermore, inhibition of PTEN with bpV(Phen), similar to ROCK inhibition, promoted cell proliferation of osteoblasts grown on PCA surface [Fig. 16C], indicating that activation of PTEN restrains cell proliferation. Pharmacologically inhibiting both PTEN and ROCK together increased cell proliferation more than treating cells with PTEN inhibitor only [Fig. 16C]. In contrast, overexpression of PTEN with GFP-PTEN plasmid in osteoblasts cultured on PCA surface decreased Akt phosphorylation levels [Fig. 16D].

Next, the control of PTEN activity was examined by its two upstream regulators, RhoA and ROCK. Overexpression of dominant negative RhoA decreased PTEN activity in MC3T3-E1 osteoblast cells on the PCA surface, whereas transfection of constitutively active RhoA increased PTEN activity [Fig. 16E]. In addition, treatment of cells with si-RhoA to knockdown endogenous RhoA expression in osteoblasts decreased PTEN activity [Fig. 16F]. Finally, ROCK inhibition using Y-27632 also

decreased PTEN activation of cells on the PCA surface [Fig. 16G]. These results indicate that proliferation of osteoblasts grown on the PCA surface is restrained by the activated RhoA/ROCK/PTEN signaling pathway.

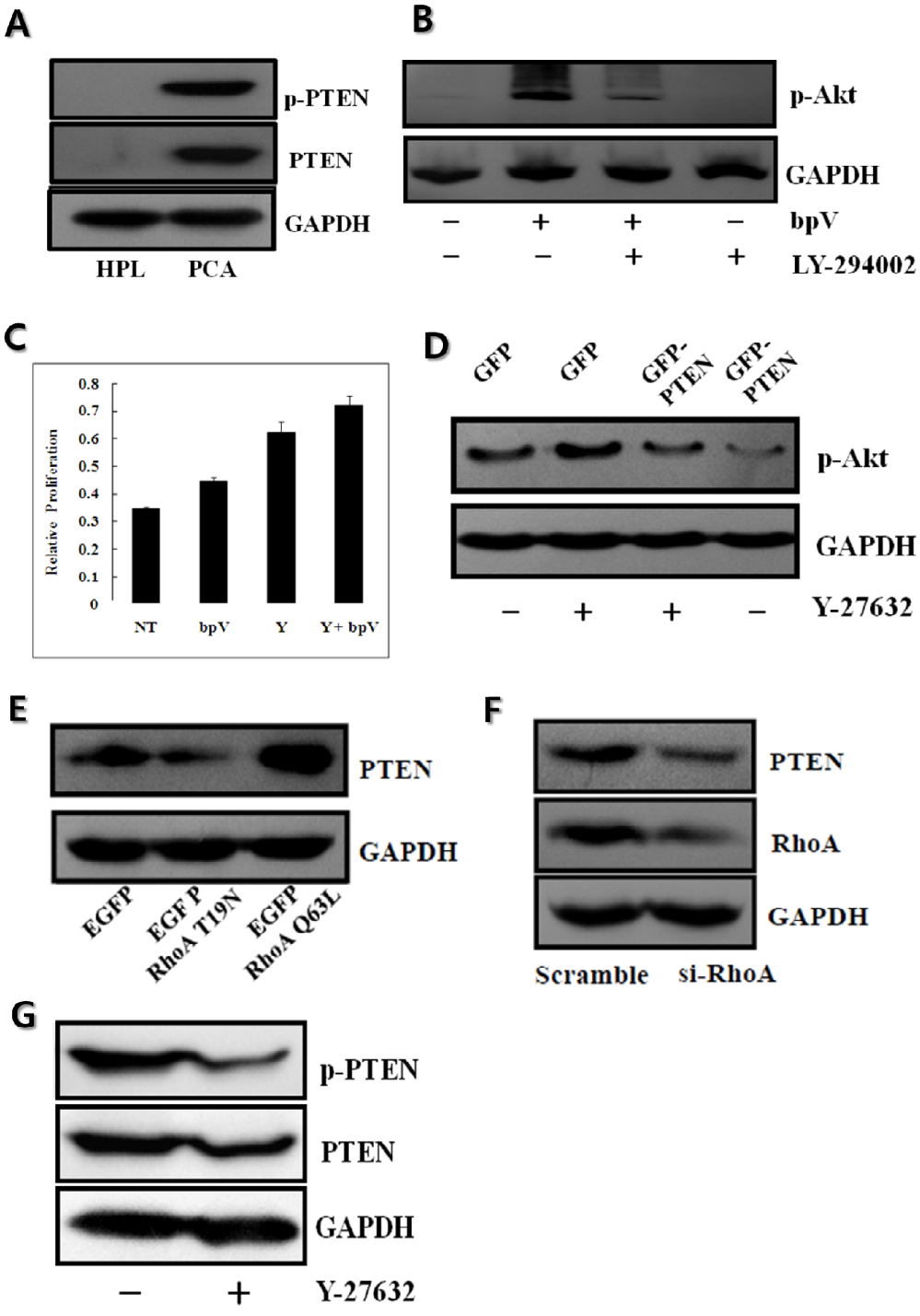


Fig. 16 PTEN leads to decreased Akt activity and cell proliferation in

MC3T3-E1 osteoblasts grown on PCA surface.

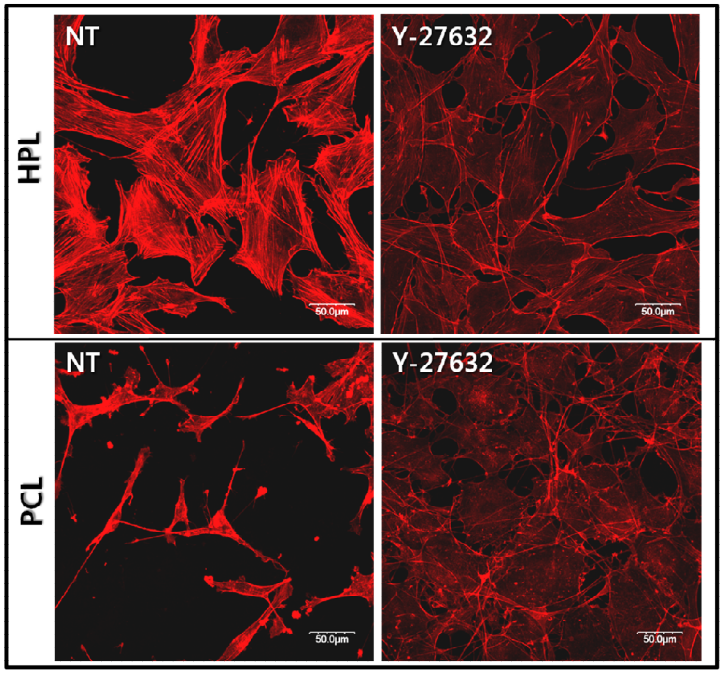
(A) Expression and phosphorylation of PTEN measured by immunoblotting cells grown for two days on HPL or PCA surfaces. (B) Effects of PTEN and PI3K inhibitions on Akt phosphorylation on osteoblasts grown on PCA surface. For 3 h, PTEN inhibitor bpV(phen) (500 nM) and/or PI3K inhibitor LY-294002 (10 μM) were added into the culture media before harvesting cells for immunoblotting. Growth factor receptor was activated with FGF-2 (50 ng/ml) for 5 min before cell lysis. (C) Effects of PTEN inhibition on cell proliferation. Cells were treated with bpV(phen) (500 nM), and/or Y-27632 (10 μM) for three days or were non-treated (NT). Data expressed as mean±SD. n=4 culture dishes. The p-value was less than 0.05 when any two groups were compared. (D) Effects of PTEN overexpression on Akt phosphorylation and its modulation by ROCK inhibition of osteoblasts grown on the PCA surface. GFP-PTEN was transiently transfected a day before cell harvest. ROCK was inhibited by adding Y-27632 (10 μM) to the culture

medium 3 h before harvesting cells for immunoblotting. Growth factor receptor was activated with FGF-2 (50 ng/ml) for 5 min before harvesting cell lysates. (E) Effects of overexpression of dominant negative or constitutively active RhoA on PTEN expression in cells grown on PCA. Cells were transiently transfected with pCDNA3-EGFP, pCDNA3-EGFP-RhoA T19N (dominant negative), or pCDNA3-EGFP-RhoA Q63L (constitutively active) and analyzed by immunoblot after 24 h. (F) PTEN and RhoA expression in osteoblasts grown on PCA for 48 h after transfection with control siRNA (scramble) or with siRNA targeting RhoA (si-RhoA). (G) ROCK inhibition decreases PTEN levels in cells grown on PCA. Expression and phosphorylation levels of PTEN were measured by immunoblotting after harvest of osteoblasts treated with and without Y-27632 (10 μM) for 24 h. GAPDH was monitored for equal loading of samples.

4. Cells on hydrophobic POLY -ε-CAPROLACTONE

PCL is one of the major scaffolds used for tissue engineering. PCL highly displays hydrophobic features similar to most of the scaffolds. Abnormal cell spreading on the PCL surface was completely rescued by ROCK inhibition [Fig. 17A and B]. Increased cell activities by ROCK inhibition was also evident in MC3T3-E1 cells grown on the PCL surface similar to those of the HPB surface.

A


NT
Y-27632
HPL
NT
Y-27632
PCL
50.0μm

B

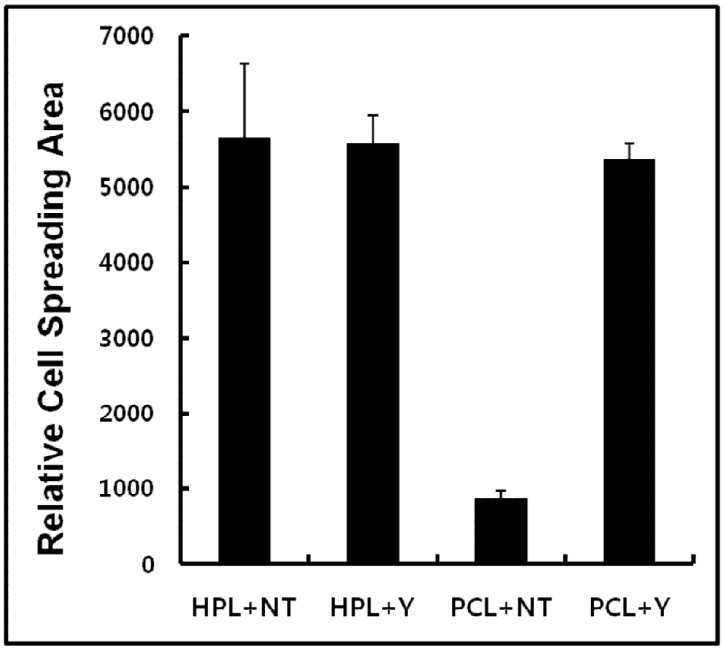

Relative Cell Spreading Area
7000
6000
5000
4000
3000
2000
1000
0
HPL+NT
HPL+Y
PCL+NT
PCL+Y

Fig. 17. ROCK inhibition by Y-27632 induces cell spreading in MC3T3-E1 cells grown on the PCL surface. The cells were incubated with or without Y-27632 on the PCL surface for 24h. The cells were stained with rhodamine-phalloidin for F-actin and the cell spreading sizes were analyzed by image J program.

IV. DISCUSSION

In the present study, it was demonstrated that the RhoA-ROCK-PTEN pathway is a molecular switch for anchorage dependence; its activity inversely correlates with the ability of cells to grow on suboptimal surfaces. When cells were poorly attached to the substrates such as HPB substrate and PCA substrate, the RhoA-ROCK-PTEN pathway was highly activated, downregulating AKT phosphorylation, which is required for cell proliferation [38, 39]. Thus, the ability of growth factors to stimulate cell proliferation was interrupted by the activation of the RhoA-ROCK-PTEN pathway in the cells exhibiting little adhesion signaling. To further confirm that proliferation is regulated by activation of the RhoA-ROCK-PTEN pathway under insufficient adhesion signals, cells were grown on normal cell culture dishes and it was found that this pathway was upregulated when the activity of the adhesion signaling associated FAK protein was inhibited. Additionally, it was confirmed that this pathway is a molecular switch for anchorage

dependence by examining the activity of this pathway in the cells grown on poly-HEMA substrates, to which cells cannot attach. The cells suspended over poly-HEMA substrates showed a high activity of this pathway.

This study clearly showed that high ROCK activity inhibits cell proliferation; the slow cell proliferation rate was rescued by ROCK inhibition with a specific inhibitor or transfection of siRNA-targeting ROCK in the cells that adhered poorly to substrates. The molecules linking cell adhesion to ROCK activity then were explored to further elucidate the pathway controlling the anchorage dependence of cell growth. The RhoA was a strong candidate for the mediator of cell adhesion signals and ROCK activation because it is a well-known upstream activator of ROCK [52, 58], and previous studies showed that RhoA activity is high when cell adhesion or integrin activation is poor [59-61]. Concomitant with the assumption, RhoA activity was dependent on adhesion strength. Its activity was higher in the cells that attached

poorly to HPB or PCA substrates. Furthermore, cell behaviors changed similarly when either RhoA or ROCK was inhibited. AKT phosphorylation and cell proliferation, which had both been decreased in the cells that were poorly attached to substrates, were increased when either RhoA or ROCK was inhibited. Downregulating RhoA activity by the transfection of siRNA-targeting RhoA, the transfection of a dominant negative RhoA construct, or the treatment of the cells with the RhoA inhibitor C3 all rescued proliferation in the cells that were grown on the poorly adherent substrates. These results indicate that RhoA is upstream of ROCK in a molecular switch that is dependent on cell adhesion strength and regulates cell proliferation rates. Recently, the RhoA-ROCK module has been suggested to control cellular functions such as adhesion, migration, proliferation and differentiation in addition to its well-known role in organizing the cytoskeleton[14, 41, 42].

To further investigate the relationship between adhesion strength and signal transduction, PTEN was focused on as a downstream effector

of the RhoA-ROCK pathway. PTEN which is a tumor suppressor downregulates the activity of AKT, which controls cell proliferation, apoptosis and migration [38, 39, 62-64]. PTEN is associated with control of the cell cycle; it is a well-known AKT regulator and a tumor suppressor that is mutated in various tumors [65-67], and it is associated with apoptotic death due to loss of cell adhesion [30, 67, 68]. ROCK has been shown to directly bind and activate PTEN to suppress inflammatory cell migration [69]. RhoA-ROCK is suggested to regulate chemotaxis by stimulating the activity of PTEN upon the formation of a RhoA-ROCK-PTEN complex [41]. Thus, the RhoA-ROCK pathway appears to regulate cell proliferation by regulating PTEN rather than other well-known downstream effectors, such as MLC, myosin light chain phosphatase, and LIM kinase, which are involved in the formation of stress fibers and increasing cellular contractility [28, 70]. Concomitant with the assumption, PTEN activity and the molecular association of ROCK and PTEN are dependent on cell adhesion strength, clearly

showing that PTEN is a member of the molecular switch for anchorage dependence. Furthermore, the increase in PTEN activity by inhibition of RhoA or ROCK activity indicates that PTEN is a downstream effector of RhoA–ROCK. Thus, AKT phosphorylation and cell proliferation can be upregulated by inhibiting PTEN as well as RhoA or ROCK.

The protein kinase B, Akt, is an important signaling molecule in cell proliferation and apoptosis[38, 39, 71]. Akt plays a role in the cell cycle progression by decreasing the expression of p21 and p27 as well as increasing cyclin D1[62, 72, 73]. In this study, Akt was used as a marker to confirm cell cycle progression when the RhoA–ROCK–PTEN pathway was inhibited in osteoblasts. RhoA activation by LPA treatment decreased Akt phosphorylation, whereas RhoA inhibition by C3 treatment increased Akt phosphorylation. Inhibiting RhoA and ROCK simultaneously increased Akt phosphorylation more than the inhibition of each molecule individually. ROCK inhibition upregulated Akt phosphorylation regardless of the activation or inhibition of RhoA which is upstream of ROCK. These

results indicate that inhibition of RhoA-ROCK-PTEN signaling increases cell proliferation by up-regulating Akt. PTEN increases intracellular levels of phosphatidylinositol-4,5-biphosphate (PIP2) by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) [43, 46, 57] and leads to the inhibition of Akt. Therefore, high activation of PTEN in cells on the PCA surface would restrict the cell cycle progression by limiting the activation of Akt through reduction of PIP3 levels. In the present study, the inhibition of RhoA-ROCK downregulated PTEN and the subsequent accumulation PIP3 by growth factor-stimulated PI3K, permitted progression and entry into the cell cycle and cell proliferation.

For upstream signaling to RhoA and ROCK, FAK was a good candidate for the link between cell adhesion and RhoA activity. FAK, which is phosphorylated at Tyr-397 upon cell adhesion [36, 37], has been shown to downregulate RhoA activity in various ways [71, 74-79]. FAK decreases RhoA activity by upregulating p190RhoGAP [74-76].

The inhibition or deletion of FAK increases RhoA activity [74-77, 79, 80]. Thus, in this study, low FAK activity may have caused the higher RhoA activity levels in the cells that adhered poorly to substrates than the cells that adhered well to substrates. An inverse relationship between level of FAK phosphorylation at Tyr-397 and RhoA activity also indicates that inadequate activation of FAK fails to suppress RhoA activity. Treating cells with inhibitors of RhoA or ROCK reversed the FAK-induced decrease in AKT phosphorylation, confirming a suppressive role of FAK on the activity of the RhoA-ROCK pathway. In addition, ROCK activation by FAK inhibition or transfection of siRNA-targeting FAK further shows an inverse relationship between FAK activity and RhoA-ROCK activity. These data indicate that RhoA-ROCK activity is increased because FAK is not activated and thus cannot downregulate RhoA activity of poorly adhering cells.

In this study, the development of actin fibers did not correlate with cell proliferation. The inhibition of the RhoA-ROCK pathway improved

cell proliferation rates without the development of stress fibers in the cells cultured on HPB or PCA substrates. These results seem to contrast with a number of reports showing that the development of actin fibers, for which the activation of the RhoA–ROCK pathway is required, is needed for cell cycle progression [24, 26, 81–83]. In addition to the effect on cell proliferation, the inhibition of the RhoA–ROCK pathway did not significantly affect the proliferation rate in the cells cultured on HPL substrates, although the actin stress fibers were disrupted. The results of this study also show that ROCK activity was upregulated when the cells were seeded on HPB or PCA substrates, cultured in suspension, or unable to transduce adhesion signals after FAK inhibition. This upregulation occurred without the development of stress fibers, although there was an upregulation of phosphorylated MLC, which is one of requirements for the development of stress fibers. These results suggest that the RhoA–ROCK pathway may control the cell cycle and the development of stress fibers separately. It is hypothesized that the

RhoA-ROCK pathway may function as a safeguard to exclude cells grown in inappropriate conditions by restricting their cellular activity rather than the development of stress fibers if cell adhesion is poor.

Calcium phosphate apatite is a typical biomaterial used as a bone substitute or a coating substrate over biomaterials, such as titanium fixtures, to enhance bone regeneration [34, 84], and on which osteoblasts differentiate [35]. A shortcoming of calcium phosphate apatite as a biomaterial, however, is the slow proliferation of osteoblasts on this surface. It has shown previously that osteoblasts do not proliferate well on the thin film of calcium phosphate apatite because growth factor signaling and adhesion signaling do not adequately cross-talk to activate the mitotic signaling pathway [5]. Even in the presence of strong growth factor stimulation, osteoblasts proliferate slowly on the calcium phosphate apatite surface where osteoblasts develop poor stress fibers and focal adhesions owing to insufficient activation of cell adhesion signaling pathways [5]. In the present study, data of this study suggest

that suppression of RhoA-ROCK-PTEN signaling pathway using pharmacological tools is an effective means to enhance cell proliferation. Slow proliferation of osteoblasts grown on the PCA surface is associated with high activation of RhoA-ROCK-PTEN pathway and the slow osteoblast proliferation was largely overcome by net down-regulation of the RhoA-ROCK-PTEN pathway using various pharmacological agents. Evidently, the physico-chemical properties of calcium phosphate apatite thin film that impede cell cycle progression and therefore their utility as a biomaterial substrate may be surmounted if intracellular signaling is properly regulated in the cultured osteoblasts. This study clearly shows that targeting intracellular signal transduction pathways through pharmacological inhibition of RhoA-ROCK-PTEN pathway rather than the surface modifications of the engineered biomaterials is a novel molecular means for controlling cell activity. The RhoA-ROCK-PTEN signaling pathway may have significant potential in tissue engineering and regenerative medicine and warrants further investigations in vivo.

V. CONCLUSION

This study clearly shows that RhoA-ROCK-PTEN activity is upregulated to downregulate the growth factor-induced phosphorylation of AKT in conditions of poor adhesion signaling as depicted in Fig 18. Suppression of this pathway that mediates the effects of cell adhesion strength on cell proliferation rescues normal proliferation rates without involving other signaling pathways. Suppressing this signaling pathway would be a simple way to manipulate anchorage-dependent cells where activity is downregulated because of inappropriate substrate conditions. Thus, low cell proliferation rates on the biomaterials such as polymers or calcium phosphate substrates used in tissue engineering can be easily upregulated by inhibiting the RhoA-ROCK-PTEN pathway. Furthermore, the elucidation of the molecular mechanism of anchorage dependence may have significant implications for understanding the behavior of cancer cells that evade anchorage dependence and for controlling cells in regenerative medicine.

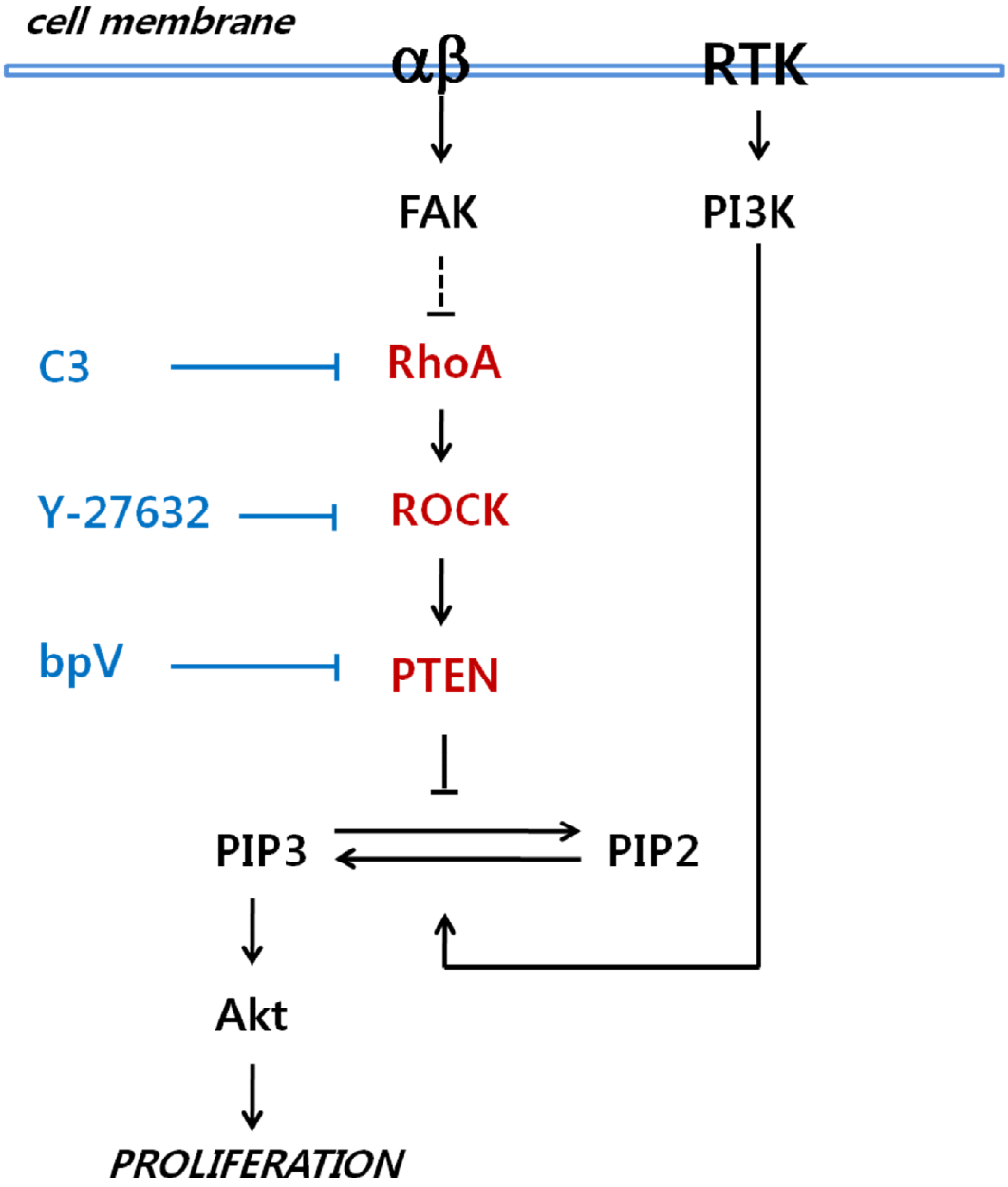


Fig. 18 A diagram illustrating how anchorage-dependent cells grown on

the substrates of poor adhesion are constrained in Akt phosphorylation by high activation of the RhoA-ROCK-PTEN pathway.

Crosstalk between growth factor receptor (receptor tyrosine kinase: RTK) and adhesion receptor (integrin; αβ) signaling through the RhoA-ROCK-PTEN pathway regulates Akt phosphorylation and cell proliferation. In the cells attached firmly to the substrates, adequate adhesion signaling downregulates RhoA-ROCK-PTEN signaling through FAK activation to permit Akt phosphorylation in response to growth factor stimulation. However, poorly adhering cells, such as those grown on the HPB or PCA substrates, fail to downregulate the RhoA-ROCK-PTEN pathway. The increased PTEN activity downregulates PIP3 levels by antagonizing the ability of PI3K to increase PIP3 levels in response to growth factor stimulation. Therefore, Akt cannot be activated to trigger cell cycle progression because PIP3 levels are decreased. However, the treatment of cells with C3, Y-27632, or bpV(Phen) can reverse the slow proliferation rate of the cells grown on the HPB substrates by inhibiting

the activities of RhoA, ROCK, or PTEN, respectively

VI. REFERANCES

1. Frisch, S.M., and Francis, H. (1994). Disruption of Epithelial Cell-Matrix Interactions Induces Apoptosis. J Cell Biol 124, 619-626.

2. Meredith, J.E., Fazeli, B., and Schwartz, M.A. (1993). The Extracellular-Matrix as a Cell-Survival Factor. Mol Biol Cell 4, 953-961.

3. Ruoslahti, E., and Reed, J.C. (1994). Anchorage Dependence, Integrins, and Apoptosis. Cell 77, 477-478.

4. Chang, E.J., Kim, H.H., Huh, J.E., Kim, I.A., Ko, J.S., Chung, C.P., and Kim, H.M. (2005). Low proliferation and high apoptosis of osteoblastic cells on hydrophobic surface are associated with defective Ras signaling. Experimental Cell Research 303, 197-206.

5. Lee, Y.J., Ko, J.S., and Kim, H.M. (2006). The role of cell signaling defects on the proliferation of osteoblasts on the calcium phosphate apatite thin film. Biomaterials 27, 3738-3744.

6. Li, H.X., Huang, D.Y., Gao, Z.X.Z., Lv, Y.H., Zhang, L.N., Cui, H.D., and Zheng, J.H. (2010). Scutellarin inhibits cell migration by regulating production of alpha(v)beta(6) integrin and E-cadherin in human tongue cancer cells. Oncol Rep 24, 1153-1160.

7. Desgrosellier, J.S., and Cheresh, D.A. (2010). Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer 10, 9-22.

8. Shanmugathasan, M., and Jothy, S. (2000). Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. Pathol Int 50, 273-279.

9. Fiucci, G., Ravid, D., Reich, R., and Liscovitch, M. (2002). Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene 21, 2365-2375.

10. Liotta, L.A., and Kohn, E. (2004). Anoikis - Cancer and the homeless cell. Nature 430, 973-974.

11. Giannoni, E., Fiaschi, T., Ramponi, G., and Chiarugi, P. (2009). Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals. Oncogene 28, 2074-2086.

12. Keledjian, K., and Kyprianou, N. (2003). Anoikis induction by quinazoline based alpha 1-adrenoceptor antagonists in prostate cancer cells: Antagonistic effect of bcl-2. J Urology 169, 1150-1156.

13. Lutolf, M.P., and Hubbell, J.A. (2005). Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 23, 47-55.

14. Tian, Y.S., Kim, H.J., and Kim, H.M. (2009). Rho-associated kinase (ROCK) inhibition reverses low cell activity on hydrophobic surfaces. Biochem Bioph Res Co 386, 499-503.

15. Frisch, S.M., and Ruoslahti, E. (1997). Integrins and anoikis. Curr Opin Cell Biol 9, 701-706.

16. Jeong, J.H., and Park, T.G. (2001). Novel polymer-DNA hybrid polymeric micelles composed of hydrophobic poly(D,L-lactic-co-glycolic acid) and hydrophilic oligonucleotides. Bioconjugate Chem 12, 917-923.

17. Mitra, S.K., Hanson, D.A., and Schlaepfer, D.D. (2005). Focal adhesion kinase: In command and control of cell motility. Nat Rev Mol Cell Bio 6, 56-68.

18. Hanks, S.K., Ryzhova, L., Shin, N.Y., and Brabek, J. (2003). Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. Front Biosci 8, D982-D996.

19. Guan, J.L., and Shalloway, D. (1992). Regulation of Focal Adhesion-Associated Protein Tyrosine Kinase by Both Cellular Adhesion and Oncogenic Transformation. Nature 358, 690-692.

20. Robinson, D.N., Ren, Y.X., Effler, J.C., Norstrom, M., Luo, T.Z., Firtel, R.A., Iglesias, P.A., and Rock, R.S. (2009). Mechanosensing through Cooperative Interactions between Myosin II and the Actin

Crosslinker Cortexillin I. Curr Biol 19, 1421-1428.

21. Miranti, C.K., and Brugge, J.S. (2002). Sensing the environment: a historical perspective on integrin signal transduction. Nat Cell Biol 4, E83-E90.

22. Zarbock, A., Kempf, T., Wollert, K.C., and Vestweber, D. (2012). Leukocyte integrin activation and deactivation: novel mechanisms of balancing inflammation. J Mol Med-Jmm 90, 353-359.

23. Bendig, G., Grimmler, M., Huttner, I.G., Wessels, G., Dahme, T., Just, S., Trano, N., Katus, H.A., Fishman, M.C., and Rottbauer, W. (2006). Integrin-linked kinase, a novel component of the cardiac mechanical stretch sensor, controls contractility in the zebrafish heart. Gene Dev 20, 2361-2372.

24. Lohez, O.D., Reynaud, C., Borel, F., Andreassen, P.R., and Margolis, R.L. (2003). Arrest of mammalian fibroblasts in G1 in response to actin inhibition is dependent on retinoblastoma pocket proteins but not on p53. J Cell Biol 161, 67-77.

25. Huang, S., and Ingber, D.E. (2002). A discrete cell cycle checkpoint in late G(1) that is cytoskeleton-dependent and MAP kinase (Erk)-independent. Experimental Cell Research 275, 255-264.

26. Hansen, L.K., Mooney, D.J., Vacanti, J.P., and Ingber, D.E. (1994). Integrin Binding and Cell Spreading on Extracellular-Matrix Act at Different Points in the Cell-Cycle to Promote Hepatocyte Growth. Mol Biol Cell 5, 967-975.

27. Munoz, C.M., van Meeteren, L.A., Post, J.A., Verkleij, A.J., Verrips, C.T., and Boonstra, J. (2002). Hydrogen peroxide inhibits cell cycle progression by inhibition of the spreading of mitotic CHO cells. Free Radical Bio Med 33, 1061-1072.

28. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271, 20246-20249.

29. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997). Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275, 1308-1311.

30. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J.H., Nakano, T., Okawa, K., et al. (1996). Regulation of myosin phosphatase by Rho and Rho-Associated kinase (Rho-kinase). Science 273, 245-248.

31. Ohashi, K., Nagata, K., Maekawa, M., Ishizaki, T., Narumiya, S., and Mizuno, K. (2000). Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. J Biol Chem 275, 3577-3582.

32. Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., et al. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nature Biotechnology 25,

681-686.

33. Folkman, J., and Moscona, A. (1978). Role of Cell-Shape in Growth-Control. Nature 273, 345-349.

34. Kim, H.M., Kim, Y., Park, S.J., Rey, C., Lee, H.M., Glimcher, M.J., and Ko, J.S. (2000). Thin film of low-crystalline calcium phosphate apatite formed at low temperature. Biomaterials 21, 1129-1134.

35. Hong, J.Y., Kim, Y.J., Lee, H.W., Lee, W.K., Ko, J.S., and Kim, H.M. (2003). Osteoblastic cell response to thin film of poorly crystalline calcium phosphate apatite formed at low temperatures. Biomaterials 24, 2977-2984.

36. Eide, B.L., Turck, C.W., and Escobedo, J.A. (1995). Identification of Tyr-397 as the Primary Site of Tyrosine Phosphorylation and Pp60(Src) Association in the Focal Adhesion Kinase, Pp125(Fak). Mol Cell Biol 15, 2819-2827.

37. Chen, H.C., Appeddu, P.A., Isoda, H., and Guan, J.L. (1996).

Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J Biol Chem 271, 26329-26334.

38. Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10, 262-267.

39. Brazil, D.P., Park, J., and Hemmings, B.A. (2002). PKB binding proteins: Getting in on the akt. Cell 111, 293-303.

40. Sahai, E., Ishizaki, T., Narumiya, S., and Treisman, R. (1999). Transformation mediated by RhoA requires activity of ROCK kinases. Curr Biol 9, 136-145.

41. Li, Z., Dong, X.M., Wang, Z.L., Liu, W.Z., Deng, N., Ding, Y., Tang, L.Y., Hla, T., Zeng, R., Li, L., et al. (2005). Regulation of PTEN by Rho small GTPases. Nat Cell Biol 7, 399-U342.

42. Vemula, S., Shi, J.J., Hanneman, P., Wei, L., and Kapur, R. (2010). ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability. Blood 115,

1785-1796.

43. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D.B., Perera, S., Roberts, T.M., and Sellers, W.R. (1999). Regulation of G(1) progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. P Natl Acad Sci USA 96, 2110-2115.

44. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W.R. (2000). Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol 20, 5010-5018.

45. Torres, J., and Pulido, R. (2001). The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus - Implications for PTEN stability to proteasome-mediated degradation. J Biol Chem 276, 993-998.

46. Cully, M., You, H., Levine, A.J., and Mak, T.W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer 6, 184-192.

47. Salmena, L., Carracedo, A., and Pandolfi, P.P. (2008). Tenets of PTEN tumor suppression. Cell 133, 403-414.

48. Gilmore, A.P., and Romer, L.H. (1996). Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. Mol Biol Cell 7, 1209-1224.

49. Agochiya, M., Brunton, V.G., Owens, D.W., Parkinson, E.K., Paraskeva, C., Keith, W.N., and Frame, M.C. (1999). Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. Oncogene 18, 5646-5653.

50. McLean, G.W., Carragher, N.O., Avizienyte, E., Evans, J., Brunton, V.G., and Frame, M.C. (2005). The role of focal-adhesion kinase in cancer. A ew therapeutic opportunity. Nat Rev Cancer 5, 505-515.

51. Abbi, S., Ueda, H., Zheng, C.H., Cooper, L.A., Zhao, J.H., Christopher, R., and Guan, J.L. (2002). Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. Mol Biol Cell

13, 3178-3191.

52. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999). Signaling from rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science 285, 895-898.

53. Kane, L.P., and Weiss, A. (2003). The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. Immunol Rev 192, 7-20.

54. Guasch, R.M., Tomas, M., Minambres, R., Valles, S., Renau-Piqueras, J., and Guerri, C. (2003). RhoA and lysophosphatidic acid are involved in the actin cytoskeleton reorganization of astrocytes exposed to ethanol. J Neurosci Res 72, 487-502.

55. Allal, C., Favre, G., Couderc, B., Salicio, S., Sixou, S., Hamilton, A.D., Sebti, S.M., Lajoie-Mazenc, I., and Pradines, A. (2000). RhoA prenylation is required for promotion of cell growth and transformation and cytoskeleton organization but not for induction

of serum response element transcription. J Biol Chem 275, 31001-31008.

56. Kapur, R., Vemula, S., Shi, J.J., Hanneman, P., and Wei, L. (2010). ROCK1 functions as a suppressor of inflammatory cell migration by regulating PTEN phosphorylation and stability. Blood 115, 1785-1796.

57. Pandolfi, P.P., Salmena, L., and Carracedo, A. (2008). Tenets of PTEN tumor suppression. Cell 133, 403-414.

58. Riento, K., and Ridley, A.J. (2003). Rocks: Multifunctional kinases in cell behaviour. Nat Rev Mol Cell Bio 4, 446-456.

59. Arthur, W.T., Petch, L.A., and Burridge, K. (2000). Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. Curr Biol 10, 719-722.

60. Sisbarro, L., Ihida-Stansbury, K., Stevens, T., Bauer, N., McMurtry, I., and Jones, P.L. (2005). The extracellular matrix microenvironment specifies pulmonary endothelial cell identity -

Roles of tenascin-C and RhoA. Chest 128, 564s-564s.

61. Ren, X.D., Kiosses, W.B., Sieg, D.J., Otey, C.A., Schlaepfer, D.D., and Schwartz, M.A. (2000). Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. J Cell Sci 113, 3673-3678.

62. Shin, I., Yakes, F.M., Rojo, F., Shin, N.Y., Bakin, A.V., Baselga, J., and Arteaga, C.L. (2002). PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med 8, 1145-1152.

63. Hemmings, B.A. (1997). Signal transduction - Akt signaling: Linking membrane events to life and death decisions. Science 275, 628-630.

64. Boni, R., Vortmeyer, A.O., Burg, G., Hofbauer, G., and Zhuang, Z. (1998). The PTEN tumour suppressor gene and malignant melanoma. Melanoma Res 8, 300-302.

65. Hopkin, K. (1998). Cancer research - A surprising function for

the PTEN tumor suppressor. Science 282, 1027-+.

66. Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 95, 29-39.

67. Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M.P., Tonks, N., and Parsons, R. (1998). The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res 58, 5667-5672.

68. Cantley, L.C., and Neel, B.G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase AKT pathway. P Natl Acad Sci USA 96, 4240-4245.

69. Vemula, S., Shi, J.J., Hanneman, P., Wei, L., and Kapur, R. (2009). ROCK1 Functions as a Suppressor of Inflammatory Cell Migration

by Regulating PTEN Phosphorylation and Stability. Blood 114, 103-103.

70. Takaishi, K., Matozaki, T., Nakano, K., and Takai, Y. (2000). Multiple downstream signalling pathways from ROCK, a target molecule of Rho small G protein, in reorganization of the actin cytoskeleton in Madin-Darby canine kidney cells. Genes Cells 5, 929-936.

71. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H.A., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231-241.

72. Kandel, E.S., Skeen, J., Majewski, N., Di Cristofano, A., Pandolfi, P.P., Feliciano, C.S., Gartel, A., and Hay, N. (2002). Activation of Akt/protein kinase B overcomes a G(2)/M cell cycle checkpoint induced by DNA damage. Mol Cell Biol 22, 7831-7841.

73. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K.,

Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R., et al. (2002). PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat Med 8, 1153-1160.

74. Holinstat, M., Knezevic, N., Broman, M., Samarel, A.M., Malik, A.B., and Mehta, D. (2006). Suppression of RhoA activity by focal adhesion kinase-induced activation of p190RhoGAP - Role in regulation of endothelial permeability. J Biol Chem 281, 2296-2305.

75. Chang, C.C., Tsai, S.Y., Lin, H., Li, H.F., Lee, Y.H., Chou, Y., Jen, C.Y., and Juan, S.H. (2009). Aryl-hydrocarbon receptor-dependent alteration of FAK/RhoA in the inhibition of HUVEC motility by 3-methylcholanthrene. Cell Mol Life Sci 66, 3193-3205.

76. Schober, M., Raghavan, S., Nikolova, M., Polak, L., Pasolli, H.A., Beggs, H.E., Reichardt, L.F., and Fuchs, E. (2007). Focal adhesion kinase modulates tension signaling to control actin and focal

adhesion dynamics. J Cell Biol 176, 667-680.

77. Raghavan, S., Vaezi, A., and Fuchs, E. (2003). A role for alpha beta 1 integrins in focal adhesion function and polarized cytoskeletal dynamics. Dev Cell 5, 415-427.

78. Chen, B., Tzen, J., Bresnick, A.R., and Chen, H. (2002). Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells. Mol Biol Cell 13, 48a-48a.

79. Lim, Y., Lim, S.T., Tomar, A., Gardel, M., Bernard-Trifilo, J.A., Chen, X.L., Uryu, S.A., Canete-Soler, R., Zhai, J., Lin, H., et al. (2008). PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility. J Cell Biol 180, 187-203.

80. Chen, B.H., Tzen, J.T.C., Bresnick, A.R., and Chen, H.C. (2002). Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null

cells. J Biol Chem 277, 33857-33863.

81. Bohmer, R.M., Scharf, E., and Assoian, R.K. (1996). Cytoskeletal integrity is required throughout the mitogen stimulation phase of the cell cycle and mediates the anchorage-dependent expression of cyclin D1. Mol Biol Cell 7, 101-111.

82. Hirano, A., and Kurimura, T. (1974). Virally Transformed-Cells and Cytochalasin-B .1. Effect of Cytochalasin-B on Cytokinesis, Karyokinesis and DNA-Synthesis in Cells. Experimental Cell Research 89, 111-120.

83. Ingber, D.E., Prusty, D., Sun, Z.Q., Betensky, H., and Wang, N. (1995). Cell shape, cytoskeletal mechanics, and cell cycle control in angiogenesis. J Biomech 28, 1471-1484.

84. Le Guehennec, L., Soueidan, A., Layrolle, P., and Amouriq, Y. (2007). Surface treatments of titanium dental implants for rapid osseointegration. Dental Materials 23, 844-854.

국문 초록

혈액에 떠있는 혈구세포나 전이 능력을 가진 종양세포를 제외한 대부분의 세포는 기질이나 인접한 세포에서 충분한 부착신호를 제공받지 못하면 충분한 성장인자 신호가 제공됨에도 불구하고 세포증식률이 감소하며, 기질로부터 완전히 분리되면 결국 죽게 되는 부착의존성(anchorage-dependency)의 특징을 보인다. 이와 같이 세포부착신호는 세포의 생존과 증식 같은 세포 활성을 유지하는데 있어서 필수적인 신호이다. 그럼에도 불구하고, 아직까지 세포의 이러한 부착의존성에 대한 분자기작은 완전히 밝혀져 있지 못한 실정이다. 본 연구는 정상 세포가 기질로부터 적절한 부착신호를 받지 못했을 경우 대표적으로 나타나는 현상인 낮은 세포 증식률과 관련하여 이러한 현상을 조절하는 분자 기전을 좀 더 근본적으로 이해하는데 목적을 두었다.

본 실험 연구를 통해 세포 증식과 같은 세포 활성도는 세포의 기질 부착 강도와 비례적으로 나타났고, 이와는 정반대로 Small GTPase RhoA (RhoA)의 활성도는 세포의 기질 부착 강도에 따라 역으로 상관 관계를 보이는 것이 확인 되었다. 이러한 RhoA 활성조절은 부착 신호가 약한 표면에서 증식하는

세포의 증식률에 서로 반비례적으로 영향을 미치는 것을 확인되었다. 추가적으로 약한 기질 부착 세포에서 높아진 RhoA 활성은 그것의 대표적인 하위 분자 (down-stream Molecule) 인 Rho-associated, coiled-coil containing protein kinase (ROCK) 의 활성을 높였고, 단계적으로 ROCK 분자의 하위 분자 중 하나인 Phosphatase and Tensin Homologue Deleted on Chromosome 10 (PTEN) 의 활성과 그것의 레벨 또한 높이고 있다는 것이 확인되었다. 요약하자면, 세포의 기질 부착강도에 따라서 흥미롭게도 RhoA, ROCK, PTEN 세포 신호 전달 축의 활성이 상당히 역동적으로 조절 되고 있었다. 특히 PTEN 는 잘 알려진 강력한 종양 억제 분자로, 일반적으로 성장인자에 의해 활성화 되는 RTK(Receptor Tyrosine Kinases)-PI3K(Phosphatidylinositol 3-kinases)-AKT 세포 신호 전달 축을 억제하여 비정상적인 세포의 생존과 증식을 조절하는 역할로 보고 되어지고 있다. 본 연구 결과를 바탕으로 부착 신호가 약한 경우 강하게 활성화 된 RhoA-ROCK-PTEN 세포 신호 전달로 인해 세포 증식에 있어 필수적이라 할 수 있는 RTK-PI3K-AKT 신호전달 축이 원활하게 작동하지

못하게 되고, 결과적으로 정상적인 세포 증식이 이루어 지지 못하고 있음을 확인하였다.

추가적으로 이러한 세포 부착 신호에 있어 RhoA 활성을 조절하는 게 아마도 Focal Adhesion Kinase (FAK) 에 의한 것으로 관찰 되었다. FAK 분자는 세포 부착 정도를 나타내어 주는 마커(Marker)로 세포가 기질에 부착 시 매개로 작용하는 integrin 에 의해 직접적으로 활성화 되는 분자(Molecule)이다. 이러한 FAK 은 이전 다른 연구 보고들에서 RhoA 의 상위 억제자 중에 하나인 p190RhoGAP 을 활성화 시켜 RhoA 를 간접적으로 억제한다고 알려져 있다. 본 연구 결과 세포와 기질의 부착이 적절히 이루어 지면 FAK 의 활성이 높아지면서 빠르게 RhoA 의 활성 낮추는 반면, 부적절한 세포-기질 부착이 이루어지는 조건에서는 충분한 FAK 의 활성이 높아지지 못해 RhoA 의 활성이 적절하게 억제 되지 하는 것이 확인 되었다.

결론적으로 RhoA-ROCK-PTEN 신호 체계를 억제하면 부착신호가 약한 표면인데도 불구하고 놀랍게도 정상표면에서의 세포와 유사한 정도까지 AKT 활성과 세포 증식이 향상 되었다. 이상의 결과들로 비추어 RhoA-ROCK-PTEN 세포 신호 전달 축은 세포의 부착 신호가 미약한 경우 강하게 활성화

되어 세포 활성을 억제하는 세포의 부착 의존성을 제어하는 분자 스위치임을 가리킨다.

주요어: 세포 부착, 부착 의존성, 세포 증식, 세포 신호 전달

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